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SIMON FLEXNER, M.D.

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THE EFFECT OF ADULT CHICKEN ORGAN GRAFTS ON THE CHICK EMBRYO.

By JAMES B. MURPHY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 1 TO 5.

(Received for publication, May 1, 1916.)

In the course of experiments on the factors of resistance to heteroplastic tissue grafts in the chick embryo, observations were made on the effects of certain organ grafts on the embryo itself.[1] The working out of the finer histological details of this process has been taken up, at my suggestion, by Dr. Vera Danchakoff. This brief note on the original observations is published now for completeness and record.

Experiments.—On the 7th day of incubation openings were made in the shells of hens' eggs containing embryos, and small amounts of finely divided adult organs were deposited on the outer membrane (allantois and chorion). After this procedure the eggs were closed and sealed with paraffin and then returned to the incubator. On the 17th or 18th day of incubation the eggs were opened for examination. This constituted a control for the experiments on heteroplastic grafting, where, in the main experiment, grafts of heterologous tissues were introduced with an addition of grafts of homologous adult organs.[2] As there were a comparatively small number of eggs with the organ grafts alone in each experiment and as the technique throughout the series was the same, the results as a whole will be considered rather than the individual experiments. The adult chicken tissues used for the experiments were spleen, liver, kidney, bone marrow, pancreas, bone, and muscle. With the exception of pancreas, which caused wide-spread digestion of the embryonic tissues, all the tissues survived the grafting. As only spleen, liver,

[1] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

[2] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.

kidney, and bone marrow caused other than local changes, the behavior of the other tissues will be disregarded in this communication.

Membranes.—On opening the eggs on the 18th day of incubation, 11 days after the introduction of a graft of one of the various organs, it was found that aside from the local changes about the graft in the outer membrane, there were scattered through the various membranes small, gray, semitranslucent nodules varying in size from a pin-point to a pin-head (Fig. 1). The majority of these contained in the center a small, firm, white dot. This change in the various membranes seemed to be no more frequent near than at a distance from the graft and occurred in other membranes of the embryo than that supporting the graft. There was considerable variation in the frequency with which this condition was observed. Its most common association was with spleen and bone marrow grafts, but it also occurred in typical form in association with the liver, and, still less frequently, with the kidney.

Histologically the nodules were made up of a collection of cells which showed every gradation from the mother cell of both the granular and non-granular white cells of the blood to the typical adult type. Every stage was found from a small clump (Fig. 2) of the former to a large dense mass with a necrotic center (Fig. 3). Immediately around the central dead area in these instances was a layer of multinuclear giant cells. Between this layer and the remaining part of the nodule was generally a zone of loose connective tissue. The outer portion was composed of a dense layer of cells of the character mentioned above.

Spleen.—Next to this change in the membranes, the most striking and constant change, after grafting with the adult organs, was observed in the embryo spleen. The spleen of the normal 18 day chick embryo is a small pinkish white organ, little larger than the head of a common pin. In embryos which bore adult organ grafts, the spleens were found to be greatly altered. This change varied considerably in extent. In some of the embryos the spleens were only slightly enlarged but showed marked congestion, and in others they were even hemorrhagic. In the extreme cases the spleen was greatly enlarged and many times the size of that seen in the normal animal (Fig. 4). The larger spleens showed numerous firm white

nodules which on the cut surface stood up above the spleen substance and presented a dry chalky appearance (Fig. 5).

The finer histology of the changes observed in the spleens, has been made the subject of a special study by Dr. Danchakoff. Hence it may suffice to say that while the spleen of a normal embryo of this age presents only a beginning differentiation of the cells, after grafting this process is well advanced and numerous cells of both the granular and non-granular type are found (Figs. 6 and 7). The firm white nodules are similar to those seen in the membranes. They consist of areas of necrosis surrounded by multinuclear giant cells with a layer of loose connective tissue separating them from the spleen substance.

Skin and Subcutaneous Tissue.—In a small proportion of embryos showing the above changes in the membranes and spleen, gross changes occurred also in the skin and subcutaneous tissues. The most marked example of this kind was from a liver graft in the embryo of which the same grayish semitranslucent nodules were scattered through the skin and subcutaneous tissues As in the other instances, the centers were frequently white and opaque (Fig. 8). Changes of this kind were perhaps more frequent than our material would suggest, but sections of the skin were taken only when macroscopic nodules were present.. The second most striking instance of this kind resulted from an adult spleen graft. This embryo showed a nodule of considerable size in the skin of the neck, and underlying this several larger masses (Fig. 9). Microscopically the nodules resembled those seen in the membranes.

Other Organ Changes.—In the liver and kidneys of the embryos clumps of cells were observed of the same type as those described above. They occurred in masses around the vessels (Figs. 10 and 11), but as a rule were not so dense as those seen in the other tissues and were free from necrosis. These changes were found in the liver and kidneys, resulting from grafts of all the tissues mentioned above; namely, spleen, bone marrow, liver, and kidney.

DISCUSSION AND SUMMARY.

The explanation of the wide-spread changes in the embryo after grafting with adult organs offers a problem of considerable interest. Just what factors are responsible for them we are unprepared to say. They occur only after the grafting of certain adult homologous organs and are absent after grafting with such tissues as the chicken sarcoma, adult muscles, bone, and cartilage. Likewise no systemic changes have been observed after grafting of various tissues from foreign species, such as rat and mouse embryos, rat and mouse tumors of various sorts, or the normal organs of an adult animal of a foreign species. Whether or not this extensive proliferation of the white blood cell elements is to be looked on as a stimulation of a center with metastasis in the membranes and organs, or whether it is a general stimulation to the anlage of the various cells, is a point difficult to determine. Judging from the distribution and character of the proliferation, the latter seems the more plausible view. Moreover, the reaction may be of the same type as that observed by Da Fano[3] in mice after immunization against cancer by means of tissue injections. The changes he describes, however, were not so pronounced in character and were for the most part confined to the plasma cell in the connective tissues. The possibility of the action of infection must also be considered. Smears from the embryo spleen and the scattered nodules failed, however, to show microorganisms, and cultures taken on the ordinary media were negative for bacteria. If the reaction was a result of bacterial invasion, we should expect to find the changes occurring after the implantation of some of the transplantable rat and mouse tumors, for it is well known that they become highly contaminated. But such is not the case. A possible explanation of the necrosis is that the nutrition supply does not keep pace with the growth, so that a condition develops analogous to that of the central necrosis in rapidly growing tumors. We conclude, therefore, that grafts of adult spleen, bone marrow, liver, and kidney placed in the outer membrane of a chick embryo cause stimulation of the embryo spleen and lead to proliferation of certain leukocytic elements in the mesoderm, subcutaneous tissues, and around vessels in the liver and kidney.

[3] Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Outer membrane of an 18 day old chick embryo inoculated with adult chicken liver on the 7th day. This shows the numerous white nodules scattered throughout the entire membrane.

FIG. 2. A small cell collection in the outer membrane of an 18 day old chick embryo after grafting with adult kidney on the 7th day of incubation. *a*, non-granular cells; *b*, granular cells.

FIG. 3. Section of a larger nodule in the embryonic membranes of a chick embryo which had received an adult spleen graft on the 7th day of incubation. *a*, dense zone of cells; *b*, loose fibrous tissue; *c*, layer of multinuclear giant cells; *d*, necrotic center.

PLATE 2.

FIG. 4. Comparison of the spleens from normal 18 day old chick embryos (*a*) with the spleens from 18 day old embryos which had received adult organ grafts on the 7th day.

FIG. 5. A large nodular spleen (*Sp.*) in an 18 day old chick embryo which had received an adult liver graft in the outer membranes on the 7th day of incubation. (*St.*, stomach.)

PLATE 3.

FIG. 6. Section of a normal spleen from an 18 day old chick embryo, showing only beginning differentiation of the cells.

FIG. 7. Spleen from an 18 day old chick embryo which had been inoculated in the outer membrane on the 7th day of incubation with adult liver. *a*, non-granular cells; *b*, granular cells.

PLATE 4.

FIG. 8. Section from the toe of an 18 day old chick embryo showing cellular masses in the skin (*a*), and in the loose subcutaneous tissues (*b*). The latter shows the central area of necrosis. This embryo had had a graft of adult liver on the 7th day of incubation.

FIG. 9. Section of skin from an 18 day old chick embryo showing a nodule in the skin (*a*), and another in the subcutaneous tissues (*b*). This embryo received an adult spleen graft on the 7th day of incubation.

PLATE 5.

FIG. 10. Kidney from an 18 day old chick embryo, showing cell collections about the vessels (*a*). A spleen graft had been introduced into the outer membrane on the 7th day of incubation.

FIG. 11. The liver from the same embryo described in Fig. 10, showing the same type of cell collections about the vessels (*a*).

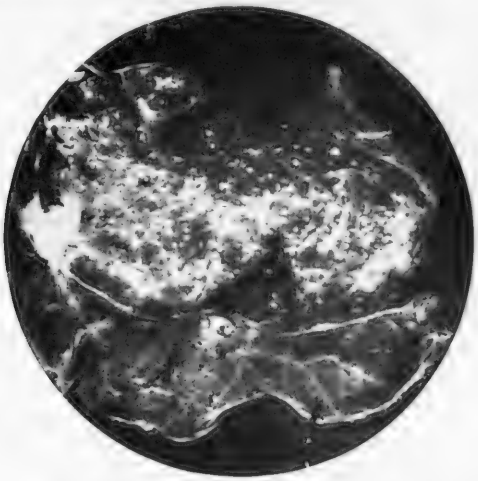


FIG. 1.

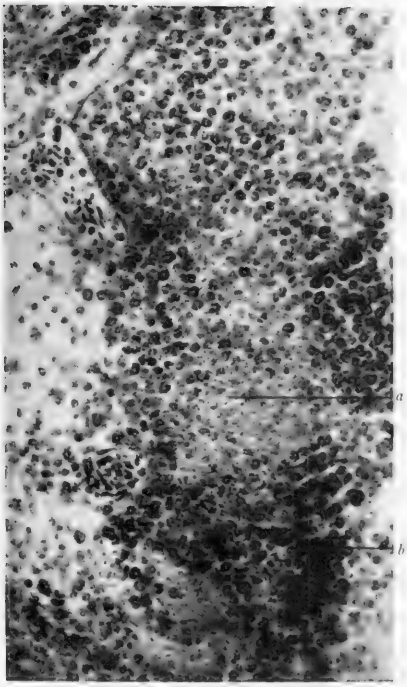


FIG. 2.

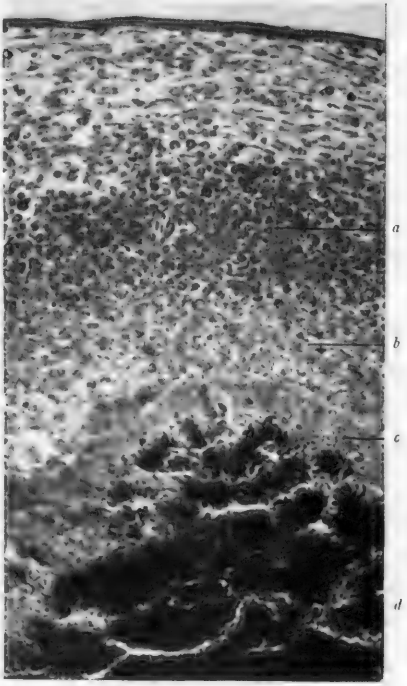


FIG. 3.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)

FIG. 4.

FIG. 5.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)

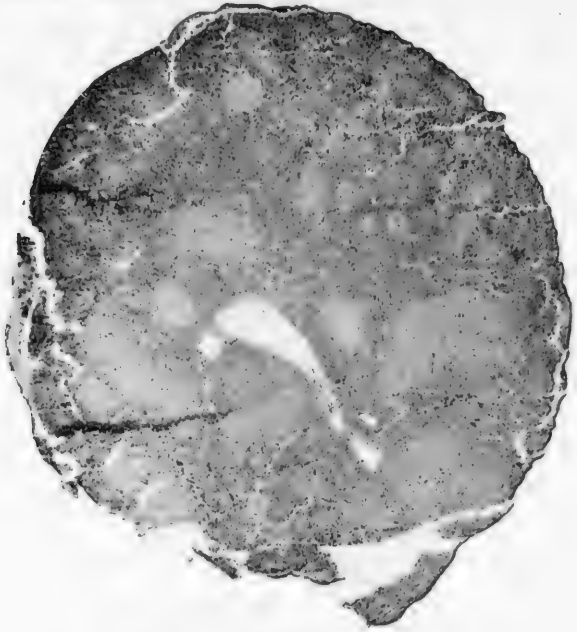


FIG. 6.

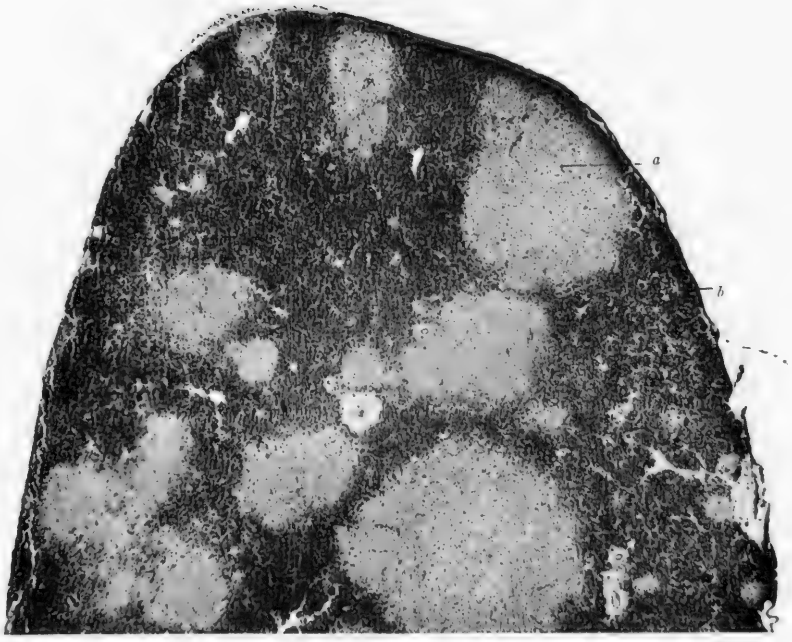


FIG. 7.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)

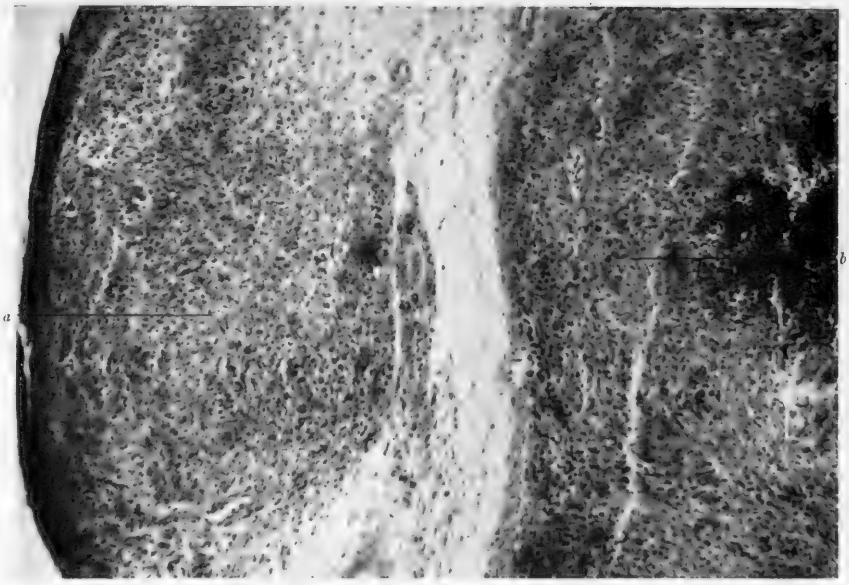


FIG. 8.

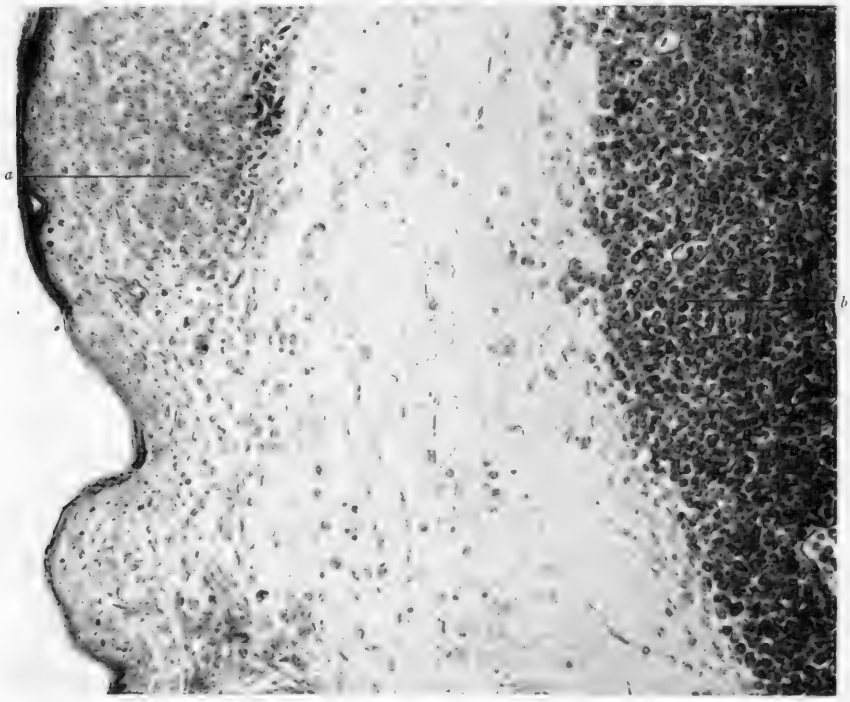


FIG. 9.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)

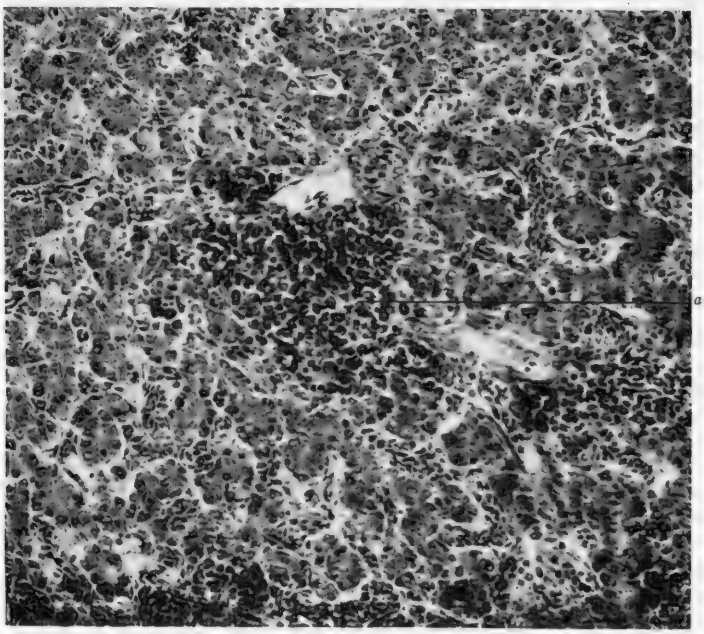


FIG. 10.

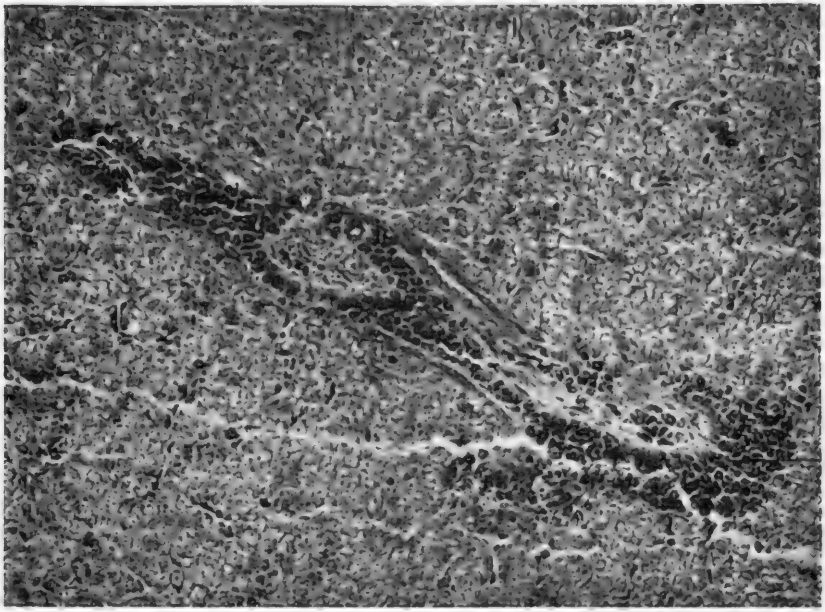


FIG. 11.

(Murphy: Adult Chicken Organ Grafts on Chick Embryo.)

IMMUNITY FACTORS IN PNEUMOCOCCUS INFECTION IN THE DOG.

By CARROLL G. BULL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 6.

(Received for publication, April 24, 1916.)

The varied native resistance that different animal species, or individuals of the same species, offer to bacterial infection has been the object of much investigation and also of speculation. This has naturally arisen from the *a priori* fact that a thorough knowledge of nature's methods of combating infections is an indispensable prerequisite of a successful treatment of infectious diseases. In our opinion, a study of the problem can be successfully approached by determining the fate of bacteria when inoculated into animals possessing widely different degrees of resistance.

Dogs have a comparatively high resistance to pneumococci, while rabbits succumb to an exceedingly small quantity of a virulent culture. When pneumococci are introduced into the circulation of rabbits, they soon begin to multiply and a fatal septicemia rapidly follows. Just what takes place in dogs under similar conditions has not been definitely determined and the mechanism of their high resistance is unknown. Hence, it was believed that a detailed investigation of the phenomena following intravenous injections of pneumococci in dogs might yield a fuller knowledge of infection and resistance in general.

Previous Observations.

Fraenkel states that dogs are quite refractory to intravenous inoculations of pneumococci.[1] Gamaléia claims that subcutaneous inoculations cause only local reactions while intrathoracic inoculations give rise to a lobar pneumonia similar

[1] Fraenkel, A., *Z. klin. Med.*, 1886, x, 401.

to that seen in man.[2] Monti showed that subdural injections of pneumococci produced typical meningitis in dogs.[3] It is claimed by Kruse and Pansini that subcutaneous inoculations of from 2 to 3 cc. of culture killed dogs within 2 to 5 days, while intravenous and intraperitoneal inoculations were without effect.[4] In a study of intrabronchial insufflations of pneumococci in dogs Lamar and Meltzer[5] observed that dogs receiving large quantities of culture developed persistent fever, did not eat, were prostrated, and died 2 to 4 days after the inoculation. These dogs gave positive blood cultures at autopsy but no detailed observations were made on the course of the septicemia. Wollstein and Meltzer[6] noted, moreover, that intrabronchial insufflations of virulent pneumococci gave rise to a bacteremia, while non-virulent cultures did not. Here, also, no quantitative observations were made.

From the literature it is readily seen that there is little definite knowledge concerning the fate of pneumococci injected into the circulation of dogs, and also that no systematic study has been made of the phenomena that the injections may incite.

The object of the present study was (1) to determine more definitely the relative resistance of dogs to pneumococcic infection, (2) to follow quantitatively the fate of the injected bacteria from the beginning to the end of the infection, and (3) to investigate the defensive mechanism and immunity responses on the part of the infected animals.

Technique.

Dogs were given intravenous inoculations of from 1 to 4 cc. per kilo of a bouillon culture of virulent[7] pneumococci. Cultures were made from the heart's blood as early as 1 minute after the injection and at short intervals for the first 6 hours and then at 24 hour periods. The blood was obtained from the heart and the cultures were made according to the method described in connection with making blood cultures from rabbits infected with pneumococci.[8] After the course

[2] Gamaléia, N., *Ann. Inst. Pasteur*, 1888, ii, 440.

[3] Monti, A., *Riforma med.*, 1889, v, 344, 350.

[4] Kruse, W., and Pansini, S., *Z. Hyg.*, 1892, xi, 279.

[5] Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

[6] Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1913, xvii, 353.

[7] The pneumococci were so virulent for rabbits that 0.00005 cc. killed within 24 hours. The virulence was maintained at this level by frequent passage in these animals.

[8] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 457.

of the infections had been established in a general way, it was found that countable plates could be made with undiluted blood after the first hour, hence measured quantities of blood were put directly into the plates and melted agar was poured in before clotting occurred. At least three plates were made from each specimen of blood and the average figures recorded in terms of the number of colonies developing from 1 cc. of blood.

Course of the Septicemia.

Through the above procedure it was discovered that the curve of the blood cultures following intravenous inoculations of pneumococci in dogs is characteristic and presents several points of interest. The injected cocci leave the circulating blood rapidly. The mass of the bacteria disappears within the first 10 minutes, the cultures dropping from about 5,000,000 colonies per cc. 1 minute after the inoculation to 175,000 at 10 minutes after. From 3 to 6 hours after the inoculations the cultures show from 50 to 200 colonies per cc. of blood. At the end of 24 hours there is, as a rule, a slight increase over the 6 hour culture. At this point the septicemia may take one of two courses. The number of organisms may gradually decrease and the blood become sterile after 2 or 3 days, a true infection not arising. On the other hand, the septicemia may gradually increase for the next 24 or 48 hours and then rapidly ascend to a climax which is attained between the 4th and 5th days. At this point a downward direction is taken and the blood becomes sterile within from 1 to 4 days. This would probably be the end of the infection if it were not for the fact that a meningitis has invariably developed in the meantime. In a number of instances, however, the blood remained sterile in the presence of a severe, and, as a rule, fatal meningitis. Every fatal case had a severe pneumococcic meningitis. The following protocols illustrate the points mentioned.

Dog 1.—Weight 6 kilos. 6 cc. of a bouillon culture of pneumococci were injected into the ear vein and observations were made as given in Table I.

Autopsy.—The lungs are normal; the heart is not injured by the punctures; no hemorrhage into the pericardium. The spleen, liver, and kidneys are congested. There are abscesses in the medullary portion of both kidneys, and films show

TABLE I.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	4,000,000	
9 "	100,000	
2 hrs.	100	Vomiting and diarrhea.
6 "	60	Drowsy.
25 "	200	Sluggish; has not eaten.
49 "	4,000	Lethargic; does not eat.
72 "	5,000	" hyperesthetic.
96 "	22,000	" "
120 "	3,000	Lying in cage; "
144 "	700	Hyperesthetic.
168 "	5	Comatose.
180 "	0	Found dead.

many diplococci and polymorphonuclear leukocytes. The subdural spaces of the brain and cord contain pus, and films show numerous pneumococci and polymorphonuclear leukocytes. The pneumococci are evenly distributed and are not being phagocyted.

In this experiment (Dog 1) we have a typical representative of a large percentage of the successfully infected dogs. The culture made 1 minute after the inoculation gave 4,000,000 colonies per cc. of blood. The pneumococci left the circulation rapidly and at the 6th hour 1 cc. of blood gave only 60 colonies. The blood cultures take a similar course in every instance and for convenience this phase of the infection will be designated as the initial drop. The initial drop was followed by a gradual increase in the number of bacteria in the blood and at the 96th hour 1 cc. gave 22,000 colonies. This proved to be the height of the blood infection and was followed by a second abrupt drop, the blood being sterile at the 180th hour. The secondary drop in this instance reminds one of the termination of pneumonia in man by lysis. The septicemia was evidently not the cause of death in this case, since at the time of death the heart's blood was sterile. On the 3rd day the dog became hyperesthetic. The hyperesthesia gradually gave way to paralysis of the extremities and this was followed by coma. Opisthotonos was also present. The autopsy revealed a severe purulent meningitis.

Dog 2.—Weight 6.5 kilos. 13 cc. of a bouillon culture of pneumococci were given intravenously. The record is given in Table II.

TABLE II.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	7,250,000	
10 "	190,000	
7 hrs.	200	2 hrs. vomiting and diarrhea.
24 "	430	Sluggish; does not eat.
48 "	1,000	" " " "
72 "	6,000	Hypersensitive.
96 "	32,000	Definite symptoms of meningitis.
120 "	0	Lying in cage; cannot stand.
144 "	0	" " " " "
168 "	0	" " " " "
192 "	0	" " " " "
216 "	10	" " " " "
240 "	0	Eats; cannot stand. Gradually recovered.

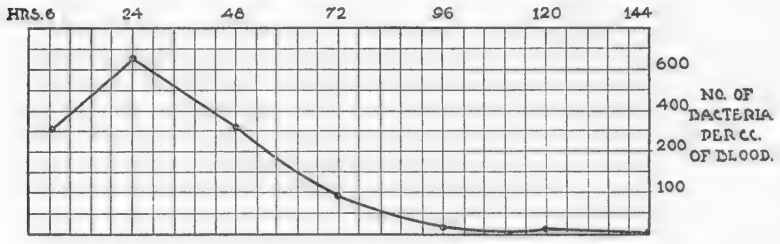
This dog differs from the first (No. 1) in that the secondary drop in the septicemia was of the nature of a crisis, going from 32,000 colonies per cc. of blood to sterility within 24 hours, with recovery. No relapse occurred in the septicemia, although a severe meningitis existed for several days. The pneumococci isolated on the 9th day grew in chains and were non-virulent (Fig. 1).

Dog 3.—Weight 7 kilos. 7 cc. of a bouillon culture of pneumococci were given intravenously. The record appears in Table III.

TABLE III.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	3,800,000	
10 "	80,000	
6 hrs.	75	2 hrs. vomiting and diarrhea.
24 "	140	Does not eat.
48 "	100	Eats.
72 "	75	Lively.
96 "	0	"
120 "	0	No further symptoms.

In Table III (Text-fig. 1) is given the course of the bacteremia when no infection develops. The initial drop occurs as in other cases. There is a slight return from the 24th to the 48th hour, but, as a rule, the blood becomes sterile before the 5th day and no further symptoms develop.



TEXT-FIG. 1. Composite curve of ten dogs that did not become infected. They were given intravenously from 1 to 3 cc. of a bouillon culture of virulent pneumococci per kilo of body weight. The abscissæ represent in hours the time after the inoculation; the ordinates, the number of pneumococci per cc. of heart's blood.

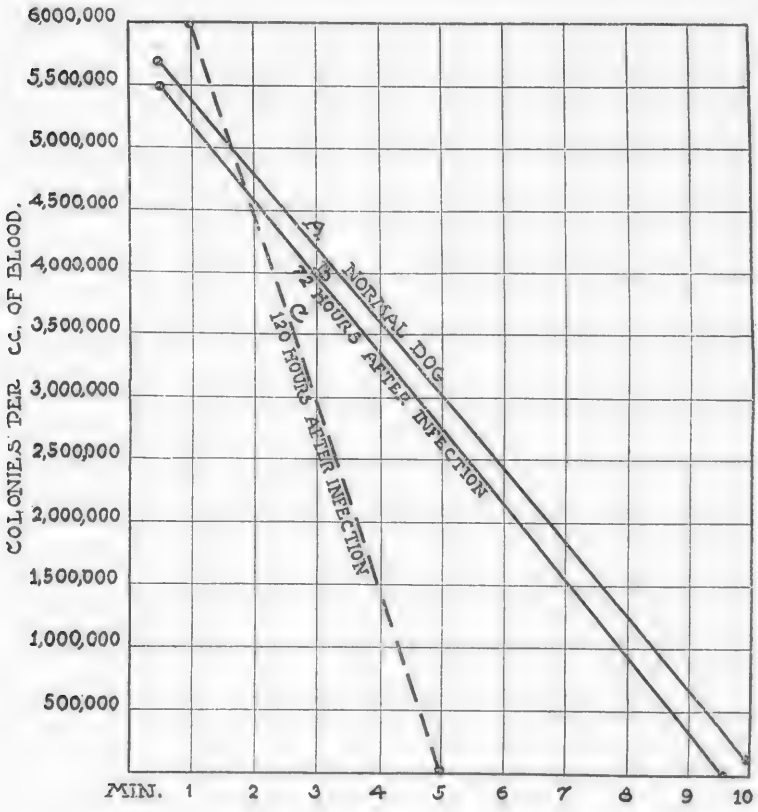
Dog 4.—Weight 6 kilos. 12 cc. of a bouillon culture of pneumococci were injected into the ear vein (Table IV).

TABLE IV.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	6,500,000	
6 hrs.	300	Drowsy.
24 "	1,500	Sluggish.
48 "	7,000	"
72 "	10,000	"
96 "	20,000	Meningeal symptoms.
120 "	4,000	Very sick.
144 "	1,300	Cannot stand.
168 "	5,000	Legs paralyzed.
192 "	35,000	Comatose.
216 "	50,000	"
240 "	110,000	"
255 "	25,000	Found dead.

Autopsy.—A severe purulent meningitis.

This protocol (Dog 4) is given because of the relapse of the septicemia. It is possible that this was due to an invasion of the blood from the meningeal infection.



TEXT-FIG. 2. Curves A, B, and C represent the rate at which the pneumococci left the circulation of dogs at the time of infection and at 72 and 120 hours after the infection, respectively.

The curves (Text-figs. 1, 2, and 3) of the blood infections as presented in the protocols have three points of especial interest: the initial drop, the ascension of the septicemia, and the secondary drop. Several questions arise concerning the nature of the forces operating

during these three phases. First, why do the blood cultures fall so rapidly immediately after the injections are made? Why does the septicemia begin first after 24 or 48 hours instead of directly after the inoculations? Are natural antibodies present and do they destroy the mass of the pneumococci, then become bound or exhausted and permit the few bacteria that escaped destruction to multiply and produce the septicemia? Or, on the other hand, may the natural antibodies not be exhausted, the fittest organisms survive and later become immune to the forces that destroyed the mass? As to the secondary drop it may be assumed as a working hypothesis that the blood suddenly acquires new forces that destroy the pneumococci or remove them from the circulation. These points are considered individually in the following pages.

Initial Drop.

Observations made in following the course of typhoid bacilli when intravenously injected into normal rabbits[9] and the effect of immune serum on pneumococcic septicemia in rabbits[8] led us to suspect that the pneumococci are agglutinated in normal dogs immediately after reaching the circulation. Indeed, experiments proved this to be the case. Pneumococci from 100 to 150 cc. of bouillon culture were injected into the ear vein and specimens of blood were taken from the heart at short intervals from the 1st to the 10th minute and examined for clumps. The observations are shown in Table V and Figs. 2 and 3.

TABLE V.

Time of taking specimen of blood.	No. of free bacteria.	No. of clumps.
min.		
1	Many.	No clumps.
2	Fewer.	Several.
3	Still fewer.	Many.
4	" "	Fewer.
5	" "	Still fewer.
6	" "	" "
7	None.	" "
8	"	" "
9	"	None found.

[9] Bull, *J. Exp. Med.*, 1915, xxii, 475.

The dog died on the 4th day.

Autopsy.—Lungs hemorrhagic; pleurisy with effusion; many pneumococci in the fluid; kidneys hemorrhagic; abscesses in the medullæ; severe meningitis.

The character of the clumps here observed differs from those formed in rabbits treated with immune serum in that they are much less compact. The agglutination proceeds more slowly also. The degree of agglutination is, however, directly proportional to the rapidity with which the blood cultures decrease. By the cultural method it was demonstrated merely that the number of viable bacteria speedily decreases immediately after the inoculations. The microscopic observations demonstrated, moreover, that the reduction in the cultures is due to the absence of the bacteria and that it is not a question of viability. An examination of the organs gave further proof of this since clumps of the bacteria were readily found.

The initial drop, then, is due to the agglutination of the pneumococci within the circulation and to the accumulation of the clumps in the lungs, liver, spleen, and other organs.

Secondary Drop.

Since the initial drop is the result of an agglutination of the pneumococci, it was logical to suppose that the secondary drop was due to a similar process. Yet, in order to explain the removal of the bacteria which had invaded the blood despite the presence of the natural agglutinins, or possibly after they had been exhausted, it was necessary to assume either that newly acquired agglutinins appear or that the pneumococci suddenly became more susceptible to the previously existing agglutinins. Hence experiments were devised to test these points.

Specimens of blood were taken from a series of dogs and the dogs were then inoculated with pneumococci in the usual way. The sera obtained before the inoculation and specimens taken each succeeding day were tested *in vitro* for agglutinins and opsonins. Neither agglutinins nor opsonins could be thus demonstrated before the 6th or 7th day. This was 48 hours after the break in the septicemia, and hence the assumption that these antibodies were operative in the animals at the time of the crisis in the septicemia seemed not to be

justified. It was thought, however, that the test could be made successfully in the animal body by determining the extent of agglutination and rate of disappearance of pneumococci injected at the time of the crisis. Thus, it was found that agglutination is more pronounced and that the bacteria leave the circulation much more rapidly at this time than even in normal dogs (Text-fig. 2). Hence a wide disparity exists between the demonstration of certain antibodies *in vitro* and *in vivo*. Representative experiments of this type are given in the protocols of Dogs 5 and 6.

Dog 5.—Weight 4.75 kilos. 14 cc. of a bouillon culture of pneumococci were given intravenously. On the 5th day after the inoculation the pneumococci from 150 cc. of a bouillon culture were injected into the ear vein, and cultures and smears were made from the heart's blood at short intervals, from the 1st to the 10th minute after the inoculation. Cultures: 1 minute, 210,000,000 per cc. of blood; 9 minutes, 2,000,000 per cc. Films: 1 minute, many free diplococci and some clumps; 3 minutes, many clumps and few free bacteria.

Dog 6.—Weight 7.75 kilos. 18 cc. of a bouillon culture of pneumococci were given intravenously. On the 5th day 10 cc. of culture were injected into the ear vein and cultures were made from the heart's blood at 1 and 8 minutes respectively. Cultures: 1 minute, 7,840,000 colonies per cc. of blood; 8 minutes, 12,500.

By comparing the rapidity with which the bacteria leave the circulation of normal dogs with that of Dogs 5 and 6, it is readily seen that the disappearance is more abrupt and complete in the latter. In normal dogs about one-twentieth of the cocci injected were still present at the expiration of 10 minutes, while in the dogs injected on the 5th day of the infection only from $\frac{1}{200}$ to $\frac{1}{600}$ of the injected bacteria were present at the expiration of 8 minutes. Since the rate at which bacteria leave the circulation of an animal is directly proportional to the extent of agglutination, we have here corroborative evidence of the microscopic finding that pneumococci are much more actively agglutinated in infected dogs at the time of the crisis in the septicemia than in normal dogs.

The results of these experiments have two points of unusual interest. First, it is shown that bacterial antibodies are effective in the animal body before they can be demonstrated *in vitro*. Second, it is demonstrated that acquired antibodies are active at the time of a crisis in pneumococcic septicemia in dogs. These facts are doubly

interesting because they seem to shed light on the cause of the crisis in pneumonia in man. Acquired antibodies do not become demonstrable *in vitro* in the serum of pneumonia patients, as a rule, until some time after the crisis,[10] but in the light of the above findings one is justified in concluding that they are operative in the patients at the time of the crisis.

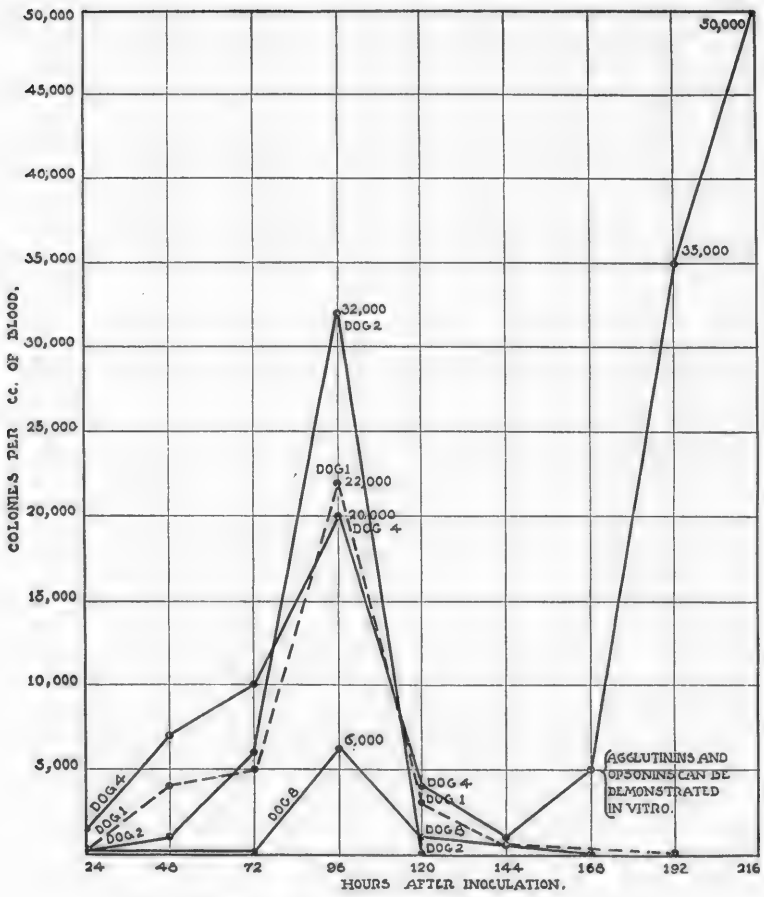
Ascension of the Septicemia.

After the initial drop the septicemia gradually increases for 48 hours and then rapidly rises to its apex (Text-fig. 3). Why a blood invasion is possible at this time, when the bacteria could not remain in the circulation at the time of the injection, is not immediately evident. Have the natural antibodies been exhausted or bound by the injected bacteria, or have the bacteria that escaped destruction become immune to the antibodies? It is possible that the septicemia is a resultant of two factors; a partial depression of the antibody activities and a lessened susceptibility on the part of the bacteria. The following experiments were made to study these points.

Bacteria.—Pneumococci were isolated from the heart's blood at each phase of the septicemia and kept on blood agar plates in the ice box until the infection had run its course. On the 6th day bouillon cultures were made from the different plates and the strains were tested for agglutination and opsonization with sera obtained from dogs on the 6th and 7th days of the infection. Thus it was found that the strains isolated as the septicemia was ascending were resistant to the action of the sera, while those isolated as the septicemia was declining were more readily opsonized and agglutinated than the original strain (Figs. 4 and 5). The resistant strains were both opsonized and agglutinated by a highly active immune horse serum, and the increased resistance disappeared after six transfers in bouillon.

Antibodies.—Dogs exhibiting a progressing septicemia were reinjected and the rate of disappearance of the newly injected pneumococci was determined.

[10] Dochez, A. R., *J. Exp. Med.*, 1912, xvi, 665.



TEXT-FIG. 3. Crisis in septicemia.

Dog 7.—Weight 8.5 kilos. 24 cc. of a bouillon culture of pneumococci were given intravenously. For blood cultures and reinjection see Table VI.

TABLE VI.

Time after inoculation.	Colonies per cc. of heart's blood.	Remarks.
1 min.	8,500,000	
6 hrs.	350	
24 "	600	
48 "	5,000	
		After the culture was made 8 cc. of culture were injected into the ear vein.
1 min. after reinjection.	3,500,000	
3 hrs. " "	3,000	
72 hrs.	14,500	
96 "	24,000	
120 "	Dog was found dead.	Meningitis.

The figures in Table VI show that even in the presence of a progressing septicemia freshly injected pneumococci rapidly leave the circulation (Text-fig. 2). Indeed, there were fewer bacteria in the heart's blood 3 hours after the second inoculation than immediately before. It was found that the number of pneumococci in the circulation 3 hours after the second injection was always in direct relation to the number present at the time the inoculation was made. As a rule, the cultures made at the expiration of 3 hours gave from 50 to 75 per cent as many colonies as those preceding the reinoculation. This held good although the dogs had infections ranging from 250 to 150,000 colonies per cc. of blood at the time they were reinoculated.

A question as to the identity of the residual pneumococci naturally arose. Are they the organisms of the septicemia, or a portion of the freshly injected culture? As already stated, cultures isolated during the ascension of the septicemia have been found to be resistant to the opsonins and agglutinins of weakly immune sera. Hence, if the residual pneumococci are the septicemic organisms, they should manifest this same resistance. This proved to be the case. Bouillon cultures were made with blood from the heart immediately before and 3 hours after the second inoculation and were tested on the following day for agglutination and opsonization with

serum taken from a dog on the 6th day after inoculation with pneumococci. The culture with which the dog was reinoculated was both opsonized and agglutinated, while the cultures obtained from the heart's blood immediately before and 3 hours after the inoculation were neither opsonized nor agglutinated.

An interpretation of the different phases of the blood infection is now possible. The initial drop is the result of the agglutination of the pneumococci in the circulation of the dogs and the accumulation of the clumps in the organs. After an incubation period of from 24 to 48 hours the pneumococci become resistant to the action of the agglutinins and possibly other antibodies and reinvade the blood stream. But before the septicemia can become fatal it is halted by the appearance of newly formed antibodies.

Severity of the Meningitis and Phagocytosis.—As previously stated, every fatal infection was accompanied by meningeal symptoms and a purulent pneumococcic meningitis was found at autopsy. A few dogs recovered after a prolonged attack of meningitis. Others, having lighter attacks and showing signs of improvement, were killed in order to study the phenomena that accompany improvement or recovery.

Films and sections from the brain and cord of the fatal cases showed large numbers of pneumococci and polymorphonuclear leukocytes. The pneumococci were evenly distributed and little or no phagocytosis had taken place. Similar preparations from dogs killed while in a state of improvement gave a different picture. The pneumococci were more or less collected into clumps and many leukocytes contained large numbers of bacteria (Figs. 6 and 7).

DISCUSSION.

The facts established in the above experiments are interesting when considered in connection with many obscure points in the epidemiology and course of infectious diseases. First, it has been shown that bacteria isolated at different stages of an infection vary in their susceptibility to specific antibodies and in their infecting power. This recalls the fact that many infectious diseases are more readily transmitted from person to person during the active phase of the dis-

ease than in early convalescence. Epidemics arise, probably, because the infectious agent is passed at short intervals from individual to individual during the early stage of the disease and, therefore, before the agent has been weakened by immunity responses on the part of the individual. As soon as early and frequent contacts are avoided by isolation, the agent becomes less infectious and the epidemic subsides.

An incubation period of about 48 hours always preceded the pneumococcic septicemia in the dogs, although millions of the bacteria were injected directly into the circulation. The experiments devised to explain this delay have shown clearly that this time is necessary for the pneumococci to adapt themselves to their new and adverse environment. When they had once become immune to the injurious antibodies that existed, a rapid multiplication occurred and symptoms of disease became manifest. And if these facts can be used to interpret the incubation period of infectious diseases in man, the logical conclusion is that it has a similar meaning there, for if bacteria find ideal conditions for multiplication on entering a new host, only a few hours will elapse between the time of infection and the appearance of symptoms of disease.

It may at first seem paradoxical that the pneumococci became more resistant to the action of antibodies in the early part of the infection and then later more susceptible. This is probably due to the nature and strength of the antibodies operative at the different phases of the infection. In the incubation period, only the natural antibodies are present, while in the decline of the septicemia acquired antibodies have appeared. Corroborative observations have been made *in vitro*. Feiler found that typhoid bacilli grown in fresh normal rabbit serum become more resistant to the bactericidal action of the serum,[11] while Stryker found that growth in highly immune horse serum renders pneumococci more agglutinable and less virulent.[12] Whether bacteria become weaker or stronger in the presence of antibodies depends, no doubt, upon the relation between the plasticity and adaptability of the bacteria and the strength of the antibodies.

[11] Feiler, M., *Z. Immunitätsforsch., Orig.*, 1916, xxiv, 411.
[12] Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.

The demonstration of the activity of newly acquired antibodies at the time of the crisis in the septicemia throws light upon the natural recovery from infections diseases in man. If the host can offer sufficient resistance to the invading bacteria to forestall a profound depression of the physiologic functions, or a fatal issue, until specific antibodies can be formed and mobilized, recovery will result. On the other hand, if the host offers little or no resistance to the bacteria at the time of infection and the incubation period is eliminated, the battle is lost before defensive antibodies can be formed. The latter condition is found in the case of pneumoccocic septicemia in rabbits and the former in pneumococcic septicemia in dogs.

The cases of meningitis offered an excellent opportunity to study the manner of the destruction of the bacteria in pneumococcic infections. Despite the fact that it has been demonstrated that agglutination, opsonization, and phagocytosis bring about the destruction of myriads of pneumococci in infected rabbits treated with immune serum,[13] it is still held that this has little or nothing to do with the destruction of the bacteria in cases of pneumonia in man.[14] One argument against the phagocytic theory is that phagocytosis is never found in the lungs of patients dying of pneumonia. What takes place in the cases that recover is, of course, undeterminable. The observations made on the acutely fatal and convalescent cases of pneumococcic meningitis in dogs indicate that the degree of phagocytosis is inversely proportional to the acuteness and severity of the infection. In the fatal cases there was very little phagocytosis, while in the convalescent cases the pneumococci were being actively phagocyted. The same condition may hold in pneumonia in man.

SUMMARY.

Intravenous inoculations of from 1 to 3 cc. per kilo of body weight of a bouillon culture of virulent pneumococci produce septicemia and meningitis in dogs.

The injected pneumococci leave the circulation rapidly, but begin to reinvade the blood from 24 to 48 hours later. The septicemia reaches its climax between the 4th and 5th days and then abruptly

[13] Bull, *J. Exp. Med.*, 1915, xxii, 466.

[14] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

declines, the blood becoming sterile within from 1 to 3 days after the height of the septicemia is reached.

The initial disappearance of the pneumococci from the circulation has been found to be due to agglutination of the diplococci in the blood stream and accumulation of the clumps in the lungs, liver, spleen, etc.

If the dogs are reinoculated during the ascension of the septicemia, the injected diplococci leave the circulation as rapidly as in normal dogs. Cultures isolated in this stage of the infection, both before and from 3 to 4 hours after the reinoculation, are resistant to the agglutinins and opsonins of immune sera that agglutinate and opsonize the cultures with which the dogs were originally infected. Thus it follows that the pneumococci are able to reinvade the circulation because they have acquired a fastness to the existing antibodies and not because the antibodies have been bound or exhausted.

By reinoculating dogs at the time of the crisis in the septicemia it has been shown that the agglutination of the pneumococci is more rapid and complete and that the diplococci leave the circulation much more rapidly than in normal dogs. Hence acquired antibodies are operative within the animals at this time although they cannot be demonstrated *in vitro* until from 24 to 48 hours later.

Pneumococci isolated as the infection is subsiding are more susceptible to the action of immune sera than the original cultures injected.

It is probable that all the dogs would have survived the infection if a meningitis had not developed.

In the acutely fatal cases of meningitis few pneumococci are phagocyted, while in the milder and convalescent cases much phagocytosis occurs.

It is suggested that the incubation period of infectious diseases is due to the fact that the infecting agents must become adapted to the adverse conditions encountered in the newly infected host before they can multiply sufficiently to produce the symptoms of disease. It is further suggested that epidemics may arise because the infectious agent is passed from person to person in the ascending stage of the disease and thus enters new hosts in a state of maximum resistance to the natural antibodies of such individuals. When early contacts

are avoided, epidemics tend to subside because the infectious agent is weakened by the action of acquired antibodies during the period of convalescence.

EXPLANATION OF PLATE 6.

FIG. 1. Pneumococci isolated from Dog 2 on the 9th day of infection. The film was made from a bouillon culture.

FIG. 2. A film from the heart's blood of a dog 30 seconds after a suspension of pneumococci had been injected.

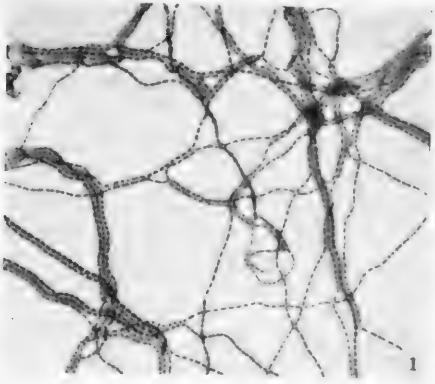
FIG. 3. A clump of pneumococci from the heart's blood of the same dog 2 minutes after the bacteria were injected.

FIG. 4. Pneumococci, immune dog serum, and guinea pig leukocytes. The bacteria were isolated from a dog 72 hours after it had been infected with pneumococci. The serum was obtained from a dog on the 7th day of a pneumococcic infection.

FIG. 5. The same as Fig. 4 except that the pneumococci were from the strain with which the above dog was infected.

FIG. 6. A film from the brain surface of a dog dying of an acute pneumococcic meningitis.

FIG. 7. A film from the brain surface of a dog in the convalescent stage of a pneumococcic meningitis.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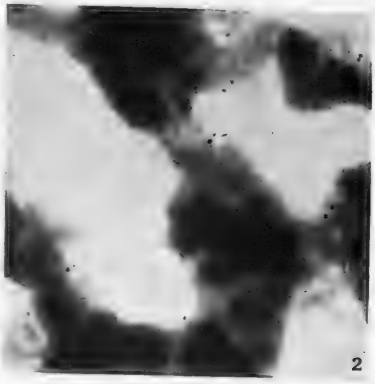
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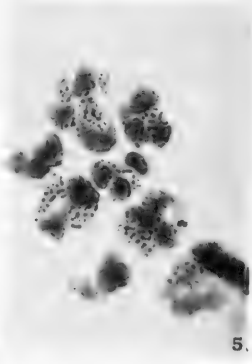
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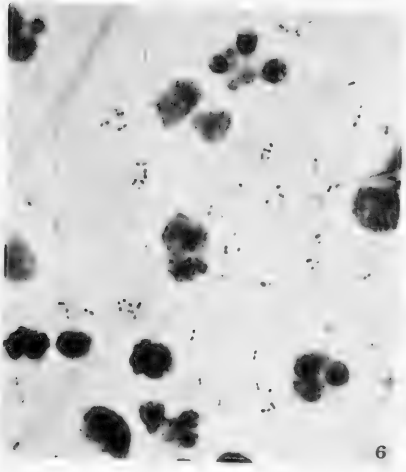
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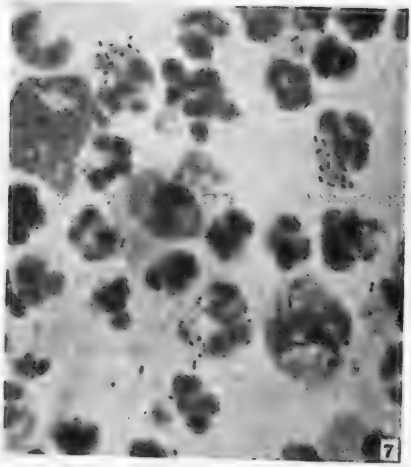
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(Bull: Immunity in Pneumococcus Infection.)

FURTHER OBSERVATIONS ON THE AGGLUTINATION OF BACTERIA IN VIVO.

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PLATE 7.

(Received for publication, April 24, 1916.)

In a previous paper[1] I reported the occurrence and the apparent significance of the agglutination of bacteria within the circulation of infected animals. The points brought out are briefly as follows: Virulent pneumococci, dysentery bacilli of the Shiga type, and virulent influenza bacilli do not agglutinate when injected into the circulation of normal rabbits; while typhoid bacilli, dysentery bacilli of the Flexner group, and non-virulent influenza bacilli are agglutinated immediately after entering the circulation of these animals. On the other hand, an intravenous injection of a small quantity of specific immune sera causes an instantaneous agglutination of pneumococci and Shiga dysentery bacilli in the circulation of infected rabbits. The bacterial clumps accumulate in the organs where they are phagocyted. It was suggested at that time that the power of the blood to cause agglutination determines, apparently, in large measure, whether the bacteria are to be promptly removed from the circulation and septicemia avoided or whether they are to remain there and produce a blood infection. Further observations have been made in this connection and are collected in this paper.

EXPERIMENTAL.

Active Immunity and Agglutination in Vivo.—As stated above, virulent pneumococci and Shiga dysentery bacilli do not agglutinate in the circulation of normal rabbits. It has been more recently observed that a strain of *Bacillus mucosus capsulatus*[2] recently isolated

[1] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 484.

[2] The author is indebted to Dr. Warren R. Sisson, of Baltimore, for this strain.

from a case of pneumonia did not agglutinate in normal rabbits. It has also been shown that pneumococci and Shiga dysentery bacilli were made readily agglutinable in normal rabbits by intravenous injections of specific immune horse serum. It was not certain, however, that these organisms would be agglutinated in actively immunized animals. Immune animals are highly resistant to infection with these bacteria and if agglutination *in vivo* plays an important part in the protection of animals against infection, it should occur in this instance. Hence rabbits were immunized with these organisms and *in vivo* agglutination tests were made. It was thus found that pneumococci, Shiga dysentery bacilli, and Friedländer bacilli are agglutinated immediately upon entering the blood stream of actively immunized animals. Indeed, the agglutination takes place so quickly and the clumps leave the circulation with such rapidity that no time can be lost in taking the blood from the heart if clumps are to be found. Only a few bacteria are left in the circulation at the end of the 1st minute.

That *Bacillus mucosus capsulatus* is agglutinated so readily in actively immune animals is especially interesting since this organism is agglutinated with difficulty *in vitro*. The sera of the rabbits used for the *in vivo* agglutination tests would not agglutinate the bacilli in dilutions above 1:5 *in vitro*.

Agglutination in Normal Rabbits.—A number of bacteria have been tested for agglutination in normal rabbits. The bacterial suspensions used in the tests were obtained either by centrifuging large quantities of bouillon cultures or by washing the bacteria from solid media with salt solution. In order to avoid false results an effort was made to free the suspensions of clumps before they were injected. As a rule, however, one specimen of blood could be obtained after the bacteria were injected before agglutination occurred, and this eliminated the possibility of the clumps that were found in the later specimens having been injected. Bacteria that agglutinate spontaneously in fluid media or in salt solution suspensions do not give satisfactory results. Sufficient quantities of the cultures were injected to give a number of organisms in each microscopic field of a film from the heart's blood.

Non-Virulent Pneumococci.—A strain of pneumococci of low virulence was obtained from The Rockefeller Institute Hospital. It had been cultivated in 10 per cent immune horse serum for seventy

generations. The immune serum was removed by ten transfers in plain bouillon. Each transfer was made by inoculating 100 cc. of bouillon with 0.5 cc. of the previous culture. This procedure diluted the immune serum so highly that it could not have influenced any of the subsequent results. Even at this time 15 cc. of a 20 hour bouillon culture did not kill a 1,500 gm. rabbit.

With strains of the same organism differing so widely in virulence, it was interesting to compare the results of intravenous inoculations with the two strains. Virulent pneumococci, it will be remembered, remain in the circulation of normal rabbits, soon begin to multiply, and a fatal septicemia develops from an exceedingly small number of organisms. The virulent organisms are neither opsonized nor agglutinated by normal rabbit serum *in vitro* or *in vivo*. The fate of the non-virulent organisms is found to be as follows: Agglutination occurs immediately after the cocci reach the circulation, the clumps leave the blood stream with great rapidity and accumulate in the organs where they are taken up by phagocytic cells. In this case no infection arises and the rabbits remain in good condition. It is also interesting to point out that the two strains do not differ in toxicity; *i.e.*, when dissolved in bile salts, one is as toxic as the other for rabbits. These two strains differ then in two essentials: the virulent strain does not agglutinate within the circulation of the normal rabbit and is not opsonized by normal rabbit serum, while the non-virulent strain agglutinates instantly after injection into the circulation of normal rabbits and is opsonized by the serum of normal animals.

Miscellaneous Strains.—The following organisms have been found to agglutinate in the circulation of normal rabbits: *Staphylococcus aureus* and *albus*, *Bacillus coli*, meningococci, gonococci, and strains of *Bacillus mucosus capsulatus* that had been on artificial media for a number of months. None of the strains used in these experiments produced general infections in rabbits and injections of subtoxic quantities were without obvious effect.

Bacillus avisepticus in Dogs and Rabbits.—As is well known, *Bacillus avisepticus*, as well as other members of the hemorrhagic septicemia group, becomes exceedingly virulent for rabbits after a few passages in these animals. Indeed, rabbits often become infected with these organisms in nature. Dogs are, on the other hand, much more resistant to infection with organisms of this group and natural

infections are not known to occur. Hence in an effort to learn more of the mechanism of the natural resistance of animals to infection, it was deemed expedient to determine the fate and behavior of these organisms when injected into the circulation. A strain of *Bacillus avisepticus* was selected for this study.

Virulence and Toxicity.—It was soon found that cultures of *Bacillus avisepticus* are highly toxic for both rabbits and dogs, but that the results are quite different in the two species if subtoxic quantities of culture are given. This is illustrated in Table I, A and B.

TABLE I.

A.

Animal.	Weight.	Time.	Injection.*	Results.
	gm.		*cc.*	
Rabbit A.	1,700	10.40 a.m. 12.05 p.m.	0.75	Died. Intoxication.
" B.	1,800	10.40 a.m. 8.30 p.m.	0.18	Found dead. More bacilli than red cells in film from heart's blood.
" C.	1,700	10.40 a.m.	0.000005	Rabbit died 17 hrs. after injection. Heavy septicemia.

* 24 hour bouillon cultures were used and all injections were intravenous.

B.

Animal.	Weight.	Time.	Injection.	Results.
	kg.	*1915*	*cc.*	
Dog A.	9.0	Oct. 16, 10.15 a.m.	1.8	
		" 16, 11.15 a.m.		Prostrate; frothy vomitus.
		" 17, 9.00 a.m.		Sluggish; lying in cage.
		" 17, 3.00 p.m.		Died. 6 colonies from 2 loops of heart's blood.
Dog B.	14.75	Oct. 18, 10.45 a.m.	0.5	No symptoms of intoxication.
		" 19, 9.00 a.m.		0.1 cc. of blood put on agar slant; no colonies developed. Dog remained in good condition.

From Table I, A it is seen that bouillon cultures of *Bacillus avisepticus* are highly toxic for rabbits, 0.5 cc. of culture per kilo causing acute death. The intoxication is largely due to a toxin, since 1 cc. per kilo of body weight of a bacteria-free filtrate from a 24 hour culture causes acute death. Rabbits B and C show that a subtoxic dose of culture causes death within a few hours by a blood infection and that a very small quantity of culture is fatal. This organism seems to grow without restraint in the circulation of rabbits.

The experiments recorded in Table I, B show that a very small quantity of culture (0.2 cc. per kilo) causes a fatal intoxication in dogs, but that a subtoxic injection is without effect. This effect is quite different from that which occurred in the rabbits and proves that cultures of *Bacillus avisepticus* are exceedingly toxic for dogs but not infectious. What is the reason or mechanism of the resistance to the infection on the part of the dogs?

Bactericidal Action of Blood and Serum.—The serum and citrated and hirudinized blood of both dogs and rabbits were tested for their bactericidal action upon *Bacillus avisepticus*. The technique was as follows: 1 cc. of the fresh undiluted serum and blood was put in a series of tubes, and 0.05 cc. of 1:40,000 dilution of a 24 hour bouillon culture of the bacilli was added. Two salt solution controls were run. One control tube was plated immediately after it was set up. The other tubes were kept at 37°C. for 2 hours and then plated.

The fresh serum and whole blood of both dogs and rabbits proved to be good culture media for the bacilli. The incubated salt solution control was sterile, but in the blood and serum tests there was a tenfold increase over the control plated at the beginning of the experiment.

Opsonins.—The Neufeld method was followed in testing the opsonic power of the sera. The bacilli were incubated with the serum for an hour before the leukocytes were added. The results are given in Table II.

Guinea pig leukocytes were used in this experiment, and, as is shown in the table, rabbit serum failed almost entirely to opsonize the bacteria for these phagocytes. It was conceivable, however, that rabbit serum would opsonize the bacilli for other phagocytes, dog or rabbit. Hence other tests were made with serum-phagocyte

TABLE II.

Dilution.	Serum.	Degree of phagocytosis.
1:2	Dog.	++++
1:10	"	++++
1:20	"	++
1:40	"	+
1:2	Rabbit.	+
1:10	"	-

combinations as follows: rabbit serum + dog phagocytes, rabbit serum + rabbit phagocytes, and guinea pig serum + dog and rabbit phagocytes. The results of all these experiments proved conclusively that the explanation of the phagocytosis was the presence of the dog serum. Any of the leukocytes would phagocyte the bacilli actively in the presence of dog serum and none of them to any degree in the presence of the other sera (Figs. 1, 2, and 3).

Agglutinins.—Rabbit serum does not agglutinate *Bacillus aviseptic us in vitro*, even in 90 per cent serum. In dog serum agglutination occurs only in the dilutions below 1:5. However, since it has been shown that bacteria agglutinate in the circulation of animals whose serum has little or no agglutinative power in the test-tube,[1] it was advisable to do the reactions *in vivo*.

Dog C.—Weight 9 kilos. The bacilli from four agar slants were suspended in salt solution and injected into the ear vein. Specimens of blood were taken from the heart, as indicated in Table III.

TABLE III.

Time after injection.	Clumps.	Free bacteria.
min.		
½	Few.	Many.
1	Many, large.	Few.
3	Some.	None.
5	None.	"

The dog was killed at the end of 30 minutes and portions of the lungs, liver, and spleen were crushed and examined for clumps. The lungs showed many large clumps and free bacilli; the liver, clumps

and a few free bacilli; the spleen, a few clumps. Leukocytes containing clumps of bacteria were found in the lung and liver specimens (Figs. 4 and 5).

The result of this experiment considered with those of other experiments indicates that these bacilli are agglutinated immediately upon entering the blood stream of dogs, and that the agglutinated bacteria accumulate in the lungs, liver, and spleen, where they are taken up by phagocytic cells.

Similar experiments were made with rabbits and it was found that the bacilli are not agglutinated, remain in the circulation, and soon begin to multiply (Fig. 6). Cultural methods showed that the injected bacilli gradually decrease in number for the 1st hour, at which time they begin to increase with marked rapidity.

DISCUSSION.

The results of the preceding experiments corroborate the occurrence and amplify the significance of the agglutination of bacteria within the circulation of infected or inoculated animals It has been pointed out that an inverse ratio seemed to exist between the power to produce septicemia and the degree of the agglutination of the infecting bacteria in the circulation of the host.[3] This conclusion was drawn from observations made with a few bacteria and with one animal species, the rabbit. The observations described above have a double import in that a large number of bacteria has been studied and the observations have been extended to other animals.

In the first place, it is shown that bacteria of low agglutinability in the test-tube are agglutinated with great facility in actively immunized animals. *Bacillus muscosus capsulatus* agglutinates with marked rapidity in the circulation of actively immunized rabbits, while the sera of the same animals do not agglutinate the bacilli in dilutions above 1:5 after several hours' incubation. Virulent pneumococci and Shiga dysentery bacilli are also readily agglutinated in the blood stream of immune rabbits. Hence it appears that active immunity insures the agglutination of the specific bacteria when they

[3] Bull, *J. Exp. Med.*, 1915, xxii, 489.

are injected into the circulation. It is possible, of course, that exceptions to this rule may be found.

The behavior of non-virulent pneumococci in the circulation of normal rabbits is especially interesting since it is so different from that of virulent strains. As far as was determined, these strains differed in only two respects: the septicemia-producing strains are neither opsonized nor agglutinated by normal rabbit serum, while the non-virulent strains are both agglutinated and opsonized, especially within the animal body. If these results are added to those obtained with such organisms as *Staphylococcus aureus* and *albus*, meningococci, gonococci, influenza bacilli, and *Bacillus coli*, the relation between agglutination and opsonization and the power to produce a general infection becomes evident; *i.e.*, they are inversely parallel.

This relation is again observed in comparing the infectiousness of *Bacillus avisepticus* for dogs and rabbits, and the fate of the bacilli when injected into the circulation of these animals. Here, also, the power of the serum to agglutinate and opsonize the bacteria was the only distinguishing feature. These results especially emphasize the significance of agglutinins and opsonins in the mechanism of natural resistance to infection, since *Bacillus avisepticus* produces a powerful toxin and is still incapable of causing a septicemia of any consequence in the presence of these antibodies.

It should be stated that we have no intention to urge that agglutinins and opsonins are all there is to the immunity forces, but we wish to emphasize the fact that they are operative within the circulation and organs of infected animals where they seem to play a decisive part in many instances.

SUMMARY.

1. Pneumococci, dysentery bacilli of the Shiga type, and *Bacillus mucosus capsulatus* are agglutinated immediately when injected into the circulation of actively immunized rabbits.

2. *Staphylococcus aureus* and *albus*, colon bacilli, meningococci, gonococci, and non-virulent pneumococci agglutinate in the circulation of normal rabbits.

3. Bouillon cultures of *Bacillus avisepticus* are highly toxic for both rabbits and dogs. The fresh sera of these animals have no bactericidal action upon the bacteria. Dog serum opsonized the bacilli *in vitro*, and they are agglutinated and opsonized in the circulation and organs of normal dogs. On the other hand, none of this occurs in connection with normal rabbits. A very small quantity of culture produces a fatal septicemia in rabbits, but a subtoxic dose is without effect in dogs.

4. The degree of agglutination and opsonization of bacteria within the animal body is inversely parallel to the infectiousness of the bacteria for the host.

EXPLANATION OF PLATE 7.

FIG. 1. *Bacillus avisepticus*, dog serum, and guinea pig leukocytes after 1 hour at 37°C.

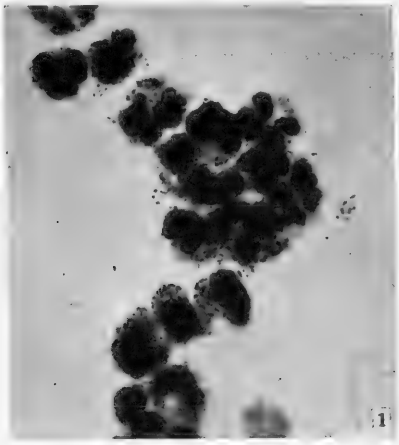
FIG. 2. *Bacillus avisepticus*, dog serum, and rabbit leukocytes after 1 hour at 37°C.

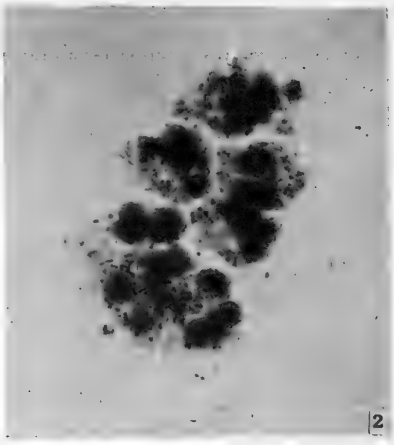
FIG. 3. *Bacillus avisepticus*, rabbit serum, and guinea pig leukocytes after 1 hour at 37°C.

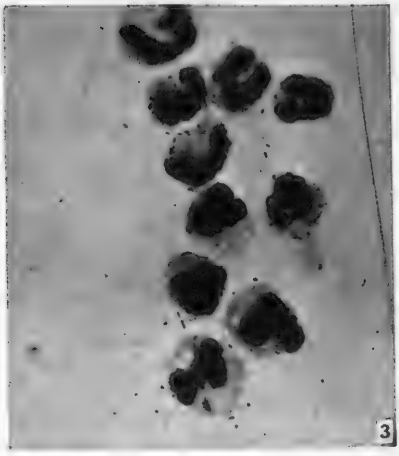
FIG. 4. A film from the heart's blood of a dog 1 minute after a suspension of *Bacillus avisepticus* had been injected into the ear vein.

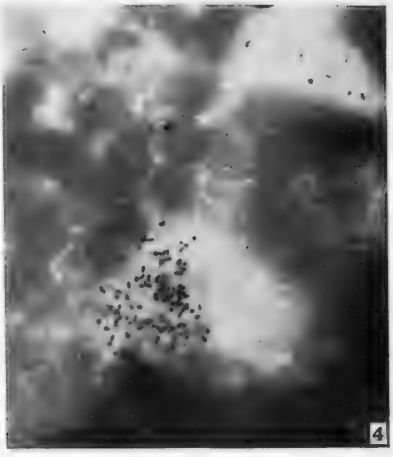
FIG. 5. Phagocytosis of *Bacillus avisepticus* by a polymorphonuclear leukocyte. The preparation is from a dog's lung, the tissue being removed 30 minutes after the bacilli were injected.

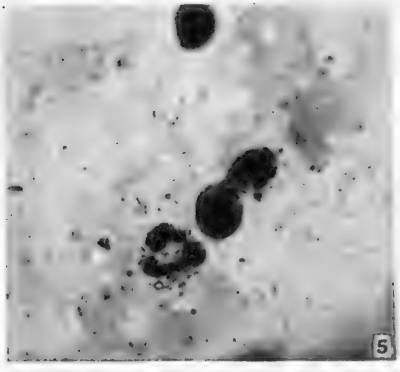
FIG. 6. A film from the heart's blood of a rabbit 5 minutes after a suspension of *Bacillus avisepticus* had been injected.

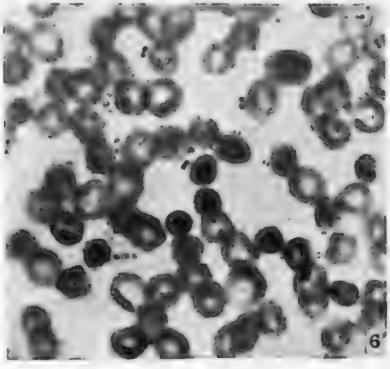












(Bull: Agglutination of Bacteria *in Vivo*.)

THE AGGLUTINABILITY OF BLOOD AND AGAR STRAINS OF TYPHOID BACILLI.

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(Received for publication, April 24, 1916.)

Gay and Claypole state that two transplantations on 10 per cent rabbit blood agar rendered typhoid bacilli non-agglutinable in an immune serum produced with plain agar cultures.[1] They further state that an immune serum obtained by means of blood agar cultures of the bacilli agglutinated equally well both blood and agar strains and that this serum agglutinated freshly isolated strains which were not agglutinated by an ordinary immune serum, thus rendering an early identification of the strains possible. They hold also that the inagglutinability of recently isolated strains is not due to some property acquired in the living animal, as a simple subculture for two generations on blood agar produces similar effects.

With a view of producing a general agglutinating serum according to this method, we first attempted to render our laboratory strains inagglutinable by growing them on 10 per cent rabbit blood agar. Two generations on blood agar failed to change the agglutinability of these strains. Cultivation on blood was continued and the agglutinability of the strains was tested after each two or three transfers. A detectable difference between the blood and agar strains did not arise, even after twenty-five generations. Since it was possible that our results were due to the particular culture with which we were working, fifty-five other cultures have been collected and subjected to a similar study.

The Strains.

We are indebted to Dr. Homer F. Swift of the Presbyterian Hospital, Dr. F. B. Humphreys of the German Hospital, and Dr. L. M.

[1] Gay, F. P., and Claypole, E. J., *Arch. Int. Med.*, 1913, xii, 621.

Famulener of St. Luke's Hospital for a number of the strains used in this work. We are especially indebted to Dr. Famulener for sending us twenty strains as they were isolated from patients. There were in all twenty-five freshly isolated strains. These were transferred to blood agar as soon as they had been identified. The remainder of the strains had been under artificial cultivation for a number of months.

Technique.

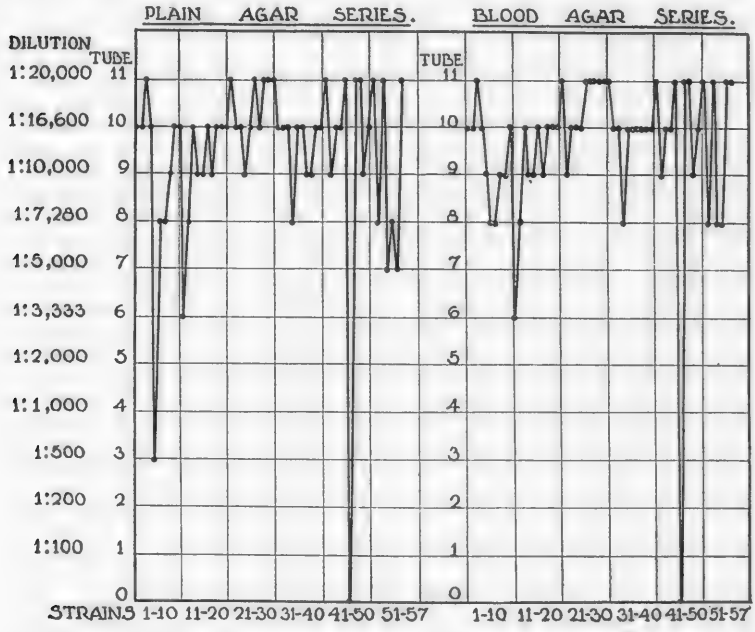
The serum used to test the agglutinability of the various strains was obtained by immunizing rabbits with a typical typhoid bacillus grown on plain agar. It agglutinated the homologous strain in a dilution of 1:20,000. The following procedure was used in making all the tests.

Graded dilutions of the serum were made with physiological salt solution. The dilutions ranged from 1:100 to 1:20,000. 1 cc. of the different dilutions was transferred to small test-tubes and one drop of bacillary emulsion was added to each, and also to a salt solution control. The emulsions of the bacilli were made by adding from 4 to 5 cc. of salt solution to 24 hour cultures on blood agar and plain agar slants, respectively. The blood and agar cultures of each strain were always tested on the same day and with the same serum dilutions. A special effort was made to have the emulsions from the corresponding blood and agar cultures of the same thickness. The tests were incubated for 2 hours at 37° C. and allowed to stand at room temperature for 2 hours before the results were read. A second reading was made the following morning. There was no difference in corresponding blood and agar cultures at the two readings. The controls were never agglutinated.

Results of the Agglutination Tests.

Fifty-seven strains were grown on blood agar for twenty-five generations and tested for agglutinability at varied intervals according to the technique just described. Only slight differences between the corresponding blood and agar strains were detected. The blood cultures were often more agglutinable than the agar cultures. There

was, however, considerable variation of the individual strains (both blood and agar cultures), and one inagglutinable strain was encountered. This strain was irregular in other particulars and will be considered more in detail below. The results are presented graphically in Text-figs. 1 and 2.

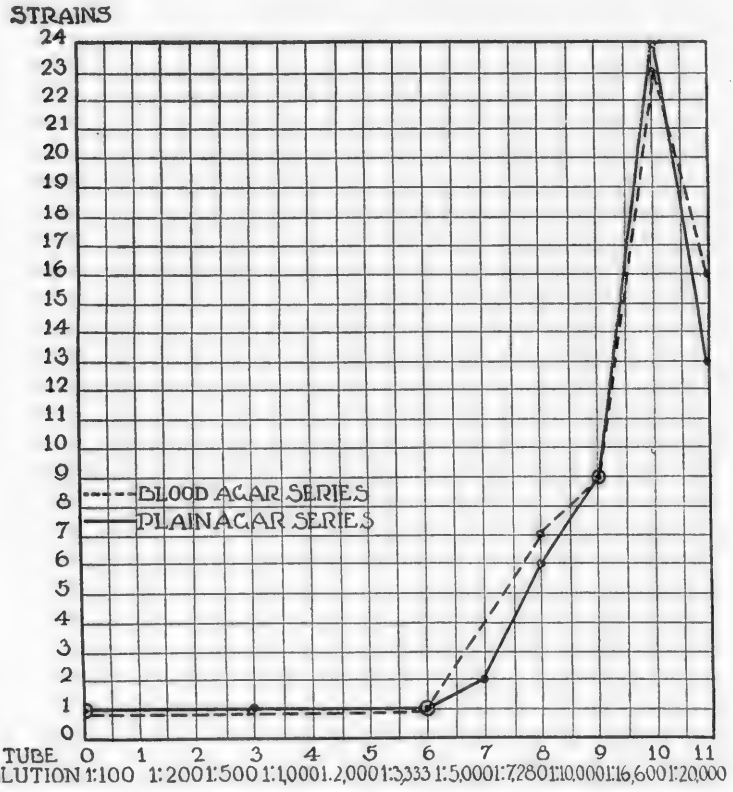


TEXT-FIG. 1. This figure shows the individual variations among the fifty-seven different strains. The dots on the abscissæ represent the individual strains from 1 to 57, in the order in which they were tested. The highest dilutions in which the strains agglutinated are represented by the ordinates.

The Irregular Strain.

This strain was brought to us by Dr. Amoss from the laboratory of Hygiene, Vermont State Board of Health. It had been recently isolated from the stool of a clinical case of typhoid and was typical according to the cultural methods ordinarily used to identify typhoid bacilli. We had not observed any irregularities in the strain until

it was found to be non-agglutinable with the immune serum used in the routine agglutination tests already described.



TEXT-FIG. 2. In this figure composite curves of the agglutinability of the blood and agar strains are given. The dilutions are represented by the abscissæ and the number of strains agglutinating in the different dilutions is represented by the ordinates.

Agglutinability.—In the routine tests the Vermont strain failed to agglutinate in a 1:100 dilution of the serum that agglutinated a number of other strains in a 1:20,000 dilution. It was later found that a dilution containing 50 per cent serum would not agglutinate

either the blood or agar cultures of this strain. It was agglutinable by fresh normal rabbit serum in a dilution of 1:5. It agglutinated in the circulation of normal rabbits as readily as other strains. A homologous serum produced by immunizing a rabbit with agar cultures agglutinated both the agar and blood cultures actively. This serum agglutinated typical strains of bacilli but not in as high dilutions as the homologous strains.

It is of interest to note the differences exhibited by the natural and acquired agglutinins. An immune serum that had stood on ice for several days failed to agglutinate the Vermont strain in 50 per cent serum, while normal rabbit serum agglutinated it in a 1:5 dilution. This indicates a lack of specificity on the part of natural agglutinins and shows further that natural agglutinins disappear spontaneously as the serum ages. The same normal serum agglutinated other strains as high as 1:20, hence the Vermont strain was naturally of lower agglutinability.

Cultural Characteristics.—In Hiss' carbohydrate serum-water media the following reactions occurred: Dextrose gave acid, no gas; levulose, acid, no gas; dextrin, acid, no gas; mannite, maltose, galactose, lactose, and saccharose gave no acid, no gas. Litmus milk gave slight acidity. Gelatin was not liquefied. Growth on potato media was typical. It produced indol as readily and as abundantly as *Bacillus coli*.

It is seen that the fermentation reactions of the Vermont strain are irregular, no acid being produced in galactose, mannite, or maltose. Control tests were run with other strains and typical reactions occurred in all the media.

The most radical discrepancy is the production of indol. This reaction has been accepted as a highly dependable test in differentiating typhoid bacilli from other closely related organisms ever since it was first used for this purpose by Kitasato.[2] Andrejew claims that a number of strains with which he worked produced indol.[3] Telle and Huber have more recently tested a number of strains and they obtained only negative results.[4] These authors believe that

[2] Kitasato, S., *Z. Hyg.*, 1889, vii, 515.

[3] Andrejew, P., *Arb. k. Gsndhtsamte.*, 1910, xxxiii, 363.

[4] Telle, H., and Huber, E., *Centr. Bakteriol., 1st Abt., Orig.*, 1911, lviii, 70.

Andrejew's results were due to faulty technique. All text-books on bacteriology teach that typhoid bacilli seldom or never produce even a trace of indol when tested by a standard technique. Therefore, the production of indol by supposed typhoid bacilli must be considered as a radical irregularity and their identity as seriously questioned.

Protection Tests.—Rabbits that have been immunized with typhoid bacilli are highly resistant to intoxication with this organism. They withstand, as a rule, from thirty to forty lethal doses of the living bacilli. The Vermont strain was highly toxic for rabbits and we decided, therefore, to test the resistance of rabbits which had been immunized with a typical strain for this strain. It was found that the rabbits would withstand from fifty to seventy-five lethal doses of the irregular strain.

The resistance of the typhoid immune rabbits to intoxication by the irregular strain either proves the typhoidal nature of the strain or indicates a marked non-specificity of the toxic substances derived from such organisms. However, the protection tests combined with cross-agglutination seem to establish the Vermont strain as a true typhoid bacillus.

SUMMARY.

Cultivation on 10 per cent rabbit blood agar did not affect the agglutinability of fifty-seven strains of typhoid bacilli.

The authors were unable to confirm the observations of Gay and Claypole on the variation in agglutinability caused by cultivating the typhoid bacillus on blood agar.

A typhoid bacillus showing irregularity in fermentation, agglutination, and indol production is described.

A FINAL REPORT ON THE CULTIVATION OF THE TUBERCLE BACILLUS FROM THE SPUTUM BY THE METHOD OF PETROFF.

By ROBERT A. KEILTY, M.D.

(From the McManes Laboratory of Pathology of the University of Pennsylvania, Philadelphia.)

(Received for publication, April 21, 1916.)

The purpose of this paper is to present a final report on the isolation of the tubercle bacillus from the sputum according to the method of Petroff.[1] From my early experience[2] it seemed to me that the method offered many opportunities, but my success at first was not great. In the present work an attempt has been made to control the possibilities which might influence a favorable or an unfavorable result. In a later communication Petroff[3] reports having isolated and cultivated the tubercle bacillus from 129 sputa out of 135 specimens. I have not been able to obtain this positive percentage, but I believe that after a little preliminary experience the results should be nearly as good.

Those accustomed to working with the tubercle bacillus realize that the results are variable, and that a method which will simplify or advance the present knowledge of tuberculosis will be of value. The method as devised by Petroff seems to open up many avenues of study in relation to the clinical course of tuberculosis and the tubercle bacillus.

According to my experience there are two important points to be realized. First, the demonstration of the tubercle bacillus unde-

[1] Petroff, S. A., A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly from the Sputum and Feces, *J. Exp. Med.*, 1915, xxi, 38.

[2] Keilty, R. A., A Study of the Cultivation of the Tubercle Bacillus Directly from the Sputum by the Method of Petroff, *J. Exp. Med.*, 1915, xxii, 612.

[3] Petroff, S. A., Some Cultural Studies on the Tubercle Bacillus, *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 276.

monstrable in sputum by other means. By microscopic examination of the cultures this is possible even though they become contaminated later by a diffuse growth of organisms. Second, the isolation of the tubercle bacillus and its study in pure culture.

The method of Petroff briefly is as follows: About 5 cc. of fresh sputum in a sterile bottle are mixed with sterile 3 per cent sodium hydroxide solution and incubated at 37°C. for 30 minutes. This should be frequently shaken and the mucin thoroughly broken up. A longer period in the incubator may be necessary for this. Place a piece of sterile litmus paper in the bottle and neutralize with sterile normal hydrochloric acid, centrifugalize at high speed for 10 minutes, and plant the sediment on three tubes of the gentian violet egg veal medium. Incubate as nearly as possible between 38° and 39°C. Variations in temperature will retard the growth decidedly.

Preparation of the Medium.—Many different modifications of egg media have been tried in the laboratory, and I find that where the whole egg is used there is not much difference. Possibly where starch is added the luxuriance of the growth is increased. From this I have lately used the medium of Petroff exclusively with some modification in the sterilization.

Meat.—500 gm. of fresh lean veal are finely ground up and infused cold over night in 500 cc. of a 15 per cent solution of glycerin in water. Squeeze out either by press or by twisting in gauze, and filter.

Eggs.—Break one dozen eggs into a large vessel and beat thoroughly. Filter through gauze. Add one part by volume of meat juice to two parts of egg.

Gentian Violet.—Add sufficient 1 per cent alcoholic solution of gentian violet to make a dilution of 1: 10,000 to one-half of the medium and tube. Tube the other half without the gentian violet.

Sterilization.—Petroff advised the sterilization of all vessels, presses, and eggs before breaking, as recommended for Dorcet's medium. It was found difficult to prevent breaks in technique, and later experience proved that absolute sterility is unnecessary.

Make up the medium in the morning after the infusion of the veal over night. Tube from 3 to 5 cc. and pack in the inspissator at about 1 p.m. Bring the temperature up to 95°C. as quickly as possible, avoiding bubbles, and keep it at this point for 1 hour. Temperatures over 100°C. will spoil the medium. Repeat the process in the inspissator at 95°C. on 2 successive days. In the early work there was considerable trouble with contamination of the medium, but with the technique carefully watched these troubles have been entirely eliminated. This should give about 125 tubes, one-half with and one-half without gentian violet. Considerable water of condensation will result. This is allowed to evaporate for a few days without dryness and the tubes are sealed as follows:

Paraffin Mixture.—Seven parts of paraffin at 55°C., one part of yellow bee's wax, and one part of petrolatum. Heat over the Bunsen as hot as possible with-

out combustion. Flame the top of the tube and cotton plug, remove the latter with sterile forceps and flame it. Immerse while burning into the hot paraffin mixture and plug, allowing the excess to drain back into the mixture. Heat the paraffin after every dozen tubes. We have never had an infection by using this method. When the tubes are in use heat before and after removing the plug. In this way tubes may be kept for as long as 8 months and frequently opened without much drying of the medium or contamination. I have had an opportunity of observing cultures obtained from many different laboratories, and find that the technique of sealing varies considerably and in some cases at least is impracticable for frequent use of the tube. For this reason and because drying was one of my earliest difficulties the method has been included in some detail.

Results.—Twenty-eight consecutive specimens were studied, most of which were kindly furnished by Dr. Baldwin Leuke, from the wards of the Philadelphia General Hospital. These were not selected in any way. They were collected during the night in sterile bottles and started the next day. The fact that they were not absolutely fresh has no doubt influenced the result somewhat. Fresh sputum as suggested by Petroff is important. In 2 of the 28 cases the tubes were broken and lost in the centrifuge and 1 was too old so that 25 cases were finally studied. Of the 25 cases, 23 were said to be advanced, 1 case, a student, undoubtedly negative, and 1 case suspected but probably negative. On examination by smear, 18 cases were positive and 7 negative for tubercle bacilli after 5 minutes' examination. Of the 18 positive cases, 12 showed cultural evidences of growth, and of the 7 negative cases, 1 showed growth. Of the 12 positive cases, 4 were obtained in pure culture and 8 showed microscopic evidence of growth; that is, colonies were too minute to be made out grossly or were overgrown by contamination, but upon microscopic examination showed groupings in numbers sufficient to exclude any doubt that they were carried over from the sputum originally. Where any doubt existed they were classed as negative. Of the 4 pure cultures, 2 averaged 10 to 14 organisms to a field upon the original sputum examination. 2 showed 1 to several fields. Of the 18 positive sputa, only 4 had many organisms to a field and 2 of these gave pure cultures, while 1 had microscopic evidence in culture and 1 had to be incubated 24 hours because of a thick ropy sputum. Of the 4 pure cultures, 1 case showed 2 contaminations out of 4 tubes inoculated. The other 3 cases grew with-

out contamination. Case 20, a negative case, was several days old when the specimen was received. 3 cases showed early microscopic evidence of growth, 1 on the 3rd day and 2 on the 4th day. This number probably would have been increased if the tubes had been examined more frequently. The first appearance of gross growth averaged 14 to 21 days. This result was dependent upon opportunities for observation and in some cases the time may be shorter. In transplants from young cultures I have frequently observed growth in 3 or 4 days and good heavy growth in 10 days on the plain egg medium.

The colonies first visible under a magnifying glass are pin-point. They dot up like minute mushrooms, tending to heap up; if scattered they will grow with umbilicated centers. They spread over one another with a typical dry hilly pattern if close together. When pure growth is first determined they should be immediately transferred to plain egg medium upon which they grow luxuriantly. They often fill the entire surface of the medium at the end of a month when they should again be transplanted. There seems to be a great variation in the rapidity of growth of different cultures. This depends upon the culture, the medium, and the incubation. The medium must be made and sterilized correctly. One batch, upon which no growth took place, was ruined by oversterilization. This is, however, an old experience with egg media. The moisture in the tube has a great deal to do with growth. The cultures will not thrive with visible water of condensation; on the other hand, they will not grow on a dry glazed surface. Finally there are unexplainable reasons for failures to grow. One series, No. 380, a subculture, was planted on ten tubes of plain egg on Jan. 23, 1916. The following note was made on Feb. 22: five tubes grew profusely, three scantily, and two were negative. All were subjected to the same conditions. For this work, therefore, it is advisable to use at least three tubes.

Contamination.—In the original planting three tubes of gentian violet medium were used. Where contaminations occurred they showed up, as a rule, at the end of 24 hours as small colonies, as diffuse, moist, smeary growths, or as localized colonies. In the majority of cases this contamination seemed to be held in check thereafter by the gentian violet; while in others the organisms continued

to grow, and in a few instances completely liquefied the medium. The contaminated cultures should be watched carefully and where growth is checked it may be possible to pick out a single colony of tubercle bacilli, transplant it, and thus obtain a pure culture. Where this is not possible, at least evidence of microscopical growth may be obtained.

Brief History of the Pure Cultures.

No. 326.—Dec. 20, 1915. Sputum incubated for 20 minutes; neutral in reaction; centrifugalized for 20 minutes at high speed.

Dec. 22. Two tubes are contaminated and discarded.

Dec. 23. A third tube shows microscopic growth with small white colonies contaminating, a large coccus. A small bit of uncontaminated material transferred to a plain egg tube No. 326 C.

Jan. 23, 1916. No. 326 C has a scattered growth in raised, dry colonies.

Feb. 5. A profuse dry growth of acid-fast bacilli. Transplanted.

Mar. 15. Culture growing profusely.

No. 328.—Dec. 20, 1915. Sputum incubated for 20 minutes; neutral in reaction; centrifugalized for 20 minutes.

Dec. 22. No growth.

Jan. 4, 1916. Shows the first evidence of growth, one small colony of pure acid-fast bacilli. Transferred to three tubes of plain egg medium, Nos. 328 A, B, and C.

Jan. 23. No. 328 C is growing well as multiple, whitish colonies tending to spread. Original tube did not continue to grow.

Mar. 15. Culture continues to grow after transplanting.

No. 364.—Jan. 6, 1916. Sputum incubated for 6 hours; slightly acid in reaction; centrifugalized 30 minutes at high speed.

Feb. 9. Two or three minute colonies the color of the medium; acid-fast bacilli in pure culture. Transplanted to plain egg medium, Nos. 364 A, B, and C.

Mar. 1. Has grown into dry, raised, granular colonies not tending to spread but to heap up.

No. 365.—Jan. 6, 1916. Sputum incubated 6 hours; slightly acid in reaction; centrifugalized for 30 minutes.

Feb. 9. Small raised colonies the color of the medium. Transplanted to plain egg medium, Nos. 365 A, B, and C.

Mar. 1. Original gentian violet tube and No. 365 C growing profusely. Nos. 365 A and B did not grow.

Swabs.—In twelve cases, Nos. 1 to 9 and 13 to 15 inclusive, sterile cotton swabs were thoroughly rubbed over the tonsils, fauces, tongue, and gums. The swabs were replaced in tubes and the latter

filled with 3 per cent sodium hydroxide. They were then incubated for 30 minutes. The sodium hydroxide was poured off except enough to cover the cotton swab. This was neutralized and the fluid centrifugalized. Both the sediment and the cotton removed from the applicator were planted on gentian violet egg tubes. The purpose in this portion of the work, the report of which is merely preliminary, is to obtain a method for the demonstration of the tubercle bacillus where sputum is not obtainable; for example, in children and in the insane.

In one instance, No. 371 from Case 14, the preliminary smear being negative, a pure culture was obtained. The history of this culture is as follows: The swab was obtained on Jan. 8, 1916; the smear from this was negative. On Jan. 17, one minute colony with pure acid-fast bacilli was present. It was transplanted to plain egg medium No. 371 X. On Mar. 15, No. 371 X has a good profuse growth of acid-fast bacilli in pure culture. The specimen of sputum from this case showed microscopic evidence of growth in 72 hours, but pure culture was not obtained owing to contamination.

This is a preliminary report as regards the swab, but it has possibilities with improved technique which will be reported on later. It may therefore be concluded that it is possible to isolate the tubercle bacillus in pure culture by Petroff's method from a swab of the throat and mouth.

As a control on the sputum work Dr. J. D. Paul of the resident staff of the Episcopal Hospital of Philadelphia examined some sputa in the laboratory of that Hospital. He selected ten cases from the dispensary service and I am indebted to him and to the Director of the Laboratory, Dr. C. Y. White, for the following report. The work was carried on entirely independently of me. From the ten cases four cases in pure culture were recovered. This gives a higher percentage of positive cases than my own work. In addition he recovered a pure culture by applying the technique to a specimen of pus from a tuberculous knee.

The method has not been studied in regard to feces, and the results with tissue have been negative as far as obtaining growth is concerned. In regard to tissue, small pieces are completely digested in the sodium hydroxide after incubation for 24 hours. From the sediment after centrifugalization the tubercle bacillus can be demonstrated

even when it is impossible or requires considerable time to demonstrate it in a section. From this result a 3 per cent sodium hydroxide solution is as good as antiformin for this purpose.

TABLE I.

Summary of Results.

No. of specimen.	No. of culture.	Date.	Type of case.	Smear.	Organisms per field.	Tubes planted.	Contaminations in 24 hrs.	Contaminations in 1 wk.	First growth.	Type of result.	Result.
		1915							*1916*		
1	326	Dec. 20	Adv.*	+	14	4	2	0	Jan. 23	Pure.	⊕
2	327	" 20	"	−	0	3	0	0			−
3	328	" 20	"	+	10	3	0	0	" 4	"	⊕
									1		
4	336	" 22	"	+	Occ.	3	1	0	Dec. 26	Micro.	+
5	337	" 22	"	+	"	3	1	0	" 26	"	+
6	338	" 22	"	+	"	3	3	0			−
		1916									
7	355	Jan. 4									
8	356	" 4									
9	357	" 4	"	−	0	3	1	0			−
									1916		
10	363	" 6	"	+	Occ.	3	0	3	Feb. 8	"	+
11	364	" 6	"	+	1	3	0	0	" 8	Pure.	⊕
12	365	" 6	"	+	Occ.	3	0	0	" 8	"	⊕
13	367	" 8	"	+	"	3	0	0	" 9	Micro.	+
14	368	" 8	"	+	"	3	2	0	Jan. 11	"	+
15	369	" 8	"	−	0	3	1	3			−
16	375	" 16	?	−	0	3	3	0			−
17	376	" 17									
18	377	" 17	Adv.	+	15	3	0	0			−
19	378	" 17	"	+	15	3	3	0	Feb. 16	"	+
20	401	Feb. 23	?	−	0	5	5	0			−
21	412	" 27	Adv.	+	Occ.	3	1	3			−
22	413	" 27	"	−	0	3	3	0			−
23	414	" 27	"	−	0	3	0	0	Mar. 23	"	+
24	415	" 27	"	+	Occ.	3	0	0	" 23	"	+
25	426	Mar. 3	"	+	"	3	0	0			−
26	427	" 3	"	+	4	3	2	0	" 23	"	+
27	428	" 3	"	+	4	3	0	0			−
28	431	" 4	"	+	Occ.	3	0	0			−

* Adv. indicates advanced; ?, suspected; Occ., occasional; Micro., microscopic evidence of growth; ⊕, pure culture.

DISCUSSION AND SUMMARY.

In summing up the practicability of the Petroff method which is in reality a refinement and improvement upon well known methods, it has possibilities both from the standpoint of diagnosis and the study of the tubercle bacillus. It is not a difficult technique, but requires considerable attention to detail. The results should be better in larger series of cases carried on in laboratories where more time is available for close observation of the cultures during the early periods of development. It is the early transplanting of minute colonies even in the presence of contaminations which results in pure cultures.

As to the practical value of the method, my experience would lead me to conclude that where sputum is obtainable in suspected cases of tuberculosis in which the tubercle bacillus cannot be demonstrated, an opportunity for diagnosis would be missed if this technique were not applied. If the result is negative it means nothing; if positive an otherwise obscure case may be cleared up. The Petroff method offers an easy opportunity of isolating large numbers of pure cultures (Table I).

CONCLUSIONS.

1. The method of Petroff offers an opportunity for the isolation of the tubercle bacillus in pure culture from sputum.
2. The method is available for the demonstration of the tubercle bacillus from sputum when otherwise not demonstrable.
3. The method is easy and rapid, but requires detail and constant observation.
4. The plain egg veal medium is the best medium thus far devised for the luxuriant growth of the tubercle bacillus.
5. The method supersedes antiformin, having all its good qualities and others in addition.
6. It is possible by applying the method to isolate the tubercle bacillus from a swab of the throat.
7. By using the digestive powers of sodium hydroxide it is possible to demonstrate more readily the presence of the tubercle bacillus in tissue, thus saving time on animal inoculation.
8. Finally, if positive results are obtained from an otherwise negative case the method opens up one more avenue of diagnosis.

VARIATIONS IN THE PNEUMOCOCCUS INDUCED BY GROWTH IN IMMUNE SERUM.

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The change produced in bacteria by growth in specific immune sera has been studied for many years. Metchnikoff (1) in 1887 found that the virulence of anthrax bacillus seemed to be diminished by growth in anti-anthrax serum. Similar results were obtained by Charrin and Roger (2) and Roger (3) with *B. pyocyaneus*, pneumococcus, and streptococcus. Later investigations, however, showed that this apparent loss of virulence was due to the protective action of the immune serum present, since bacteria, freed from the serum in which they had been grown, showed no alteration in their virulence (Metchnikoff (4), Sanarelli (5), Issaeff (6)). The study of the effect of this treatment has been confined chiefly to observations on *B. choleræ*, *B. typhosus*, and other intestinal organisms (von Ransom and Kitashima (7), Müller (8), Eisenberg (9), Walker (10), Smith and Reagh (11), and Feiler (12)). Investigators have found that bacteria grown in homologous immune sera, or in the peritoneal cavities of immune animals, show marked increase in virulence and in resistance to bactericidal action, and a loss of agglutinability. The increase in virulence and in resistance to bactericidal action would seem to indicate a biological adaptation on the part of the bacteria, similar to the process of increased resistance which the animal organism develops when invaded by infectious agents. The loss of agglutinability of the organisms treated with immune serum has been explained as being possibly attributable to an inadequate receptor apparatus, this condition having been brought about by the injurious action of the immune serum upon the organism. Joos (13) has demonstrated that such an effect is brought about by heating typhoid bacilli. He finds that the agglutinin-producing substance, agglutinogen, of typhoid bacilli apparently consists of two elements, which he designates as α- and β-agglutinogen. The α-agglutinogen is easily destroyed by heating at 60–62°C., while the β-agglutinogen is heat-resistant. Injection of living unheated bacilli produces both α- and β-agglutinins, while the injection of heated bacilli produces only β-agglutinins. Cole (14) has found that inagglutinable typhoid strains possess less power to absorb agglutinins than do normal strains, and, when injected into animals, produce sera which are not as highly agglutinating, even for the injected strain, as the sera produced by highly agglutinable strains. These experiments seem to show that the receptor apparatus of the inagglutinable strains is not so complete as that of the agglutinable bacilli.

Recently Friel (15) has found that by growing pneumococci in immune serum they became agglutinable and phagocytable in normal rabbit serum, and less virulent for mice than the untreated strains.

The present investigation was undertaken in order to study the different types of pneumococcus with regard to this phenomenon and to determine whether any variation of type organisms occurs after treatment with Antipneumococcus Serum I or II.

The experiments were made with virulent strains of the pneumococcus, freshly isolated from the heart's blood of mice dead from pneumococcus septicemia, or obtained from blood cultures from cases of lobar pneumonia. In all experiments determinations of morphology, bile solubility, inulin fermentation, capsule formation, virulence, and agglutination were made before the strain was subjected to treatment. Cultures were then made in normal and immune serum bouillon, and, in some instances, as a control, in plain bouillon. All serum media were prepared by the addition of 0.5 cc. of serum to 4.5 cc. of the nutrient beef infusion bouillon used for routine culture medium, the immune serum bouillon containing one part in ten of a highly potent antipneumococcus horse serum, the normal serum bouillon containing the same amount of normal horse serum. Cultures were made by inoculation of 0.5 cc. of a bouillon culture of the strain to be tested, the tubes were then incubated at 37°C. for 18 hours, and after that time 0.5 cc. of this serum culture was reinoculated directly into another tube of serum bouillon. In some instances cultures were transferred daily, in others at weekly intervals, being kept in the refrigerator during the intervals between incubation. Two different series of subcultures of each strain tested were employed, one being cultured consecutively in immune serum bouillon, the other in normal serum bouillon. The manner of growth and morphology of the organisms were noted each time that a culture was transferred. It was noted that all strains grew luxuriantly under the conditions and could be kept growing, by repeated transfer, for indefinite lengths of time. Two strains have now been grown under such conditions for over a year. Type strains grown in the homologous immune serum showed at first complete agglutination, the growth sedimenting after 18 hours' incubation in a hard mass which could be broken apart only with difficulty. After five or six transfers of Type

I in homologous serum, the growth sedimented in a flocculent mass which could be easily shaken apart. In order to produce the same effect with Type II organisms, twelve to fifteen transfers in immune serum were necessary. The microscopic picture was that of the well known thread reaction, individual organisms presenting a swollen appearance and growing in long chains or clump formation. All strains retained the original characteristics of bile solubility, inulin fermentation, and reaction to Gram stain. Marked differences, however, between the immune and normal serum treated organisms were noted in growth on blood media, agglutination, capsule formation, pathogenicity for white mice and rabbits, and antigenic and opsonic reactions.

Type strains which had been grown for a number of transfers in immune serum lost the characteristic moist, confluent, greenish growth on blood media and grew in dry, isolated, brownish colonies which showed a tendency to produce hemolysis of the blood cells in the media. When grown in plain bouillon subsequent to serum treatment, these strains showed marked sedimentation, this characteristic persisting even after twenty-five passages in plain bouillon. They also showed less tendency to form methemoglobin than the normal serum treated strains.

Variations in Agglutination.

The change noted was the development of a non-specific agglutinative reaction by the immune serum treated strains. Those strains which previously were agglutinable only in the homologous serum, subsequently were agglutinated somewhat less completely in this serum, and were also agglutinated by heterologous immune sera, and in some instances even by normal serum. All agglutination tests were made according to the following routine. 0.5 cc. of the 1:10 serum culture was inoculated into 5 cc. of plain bouillon; after this subculture had grown from 4 to 6 hours, 0.5 cc. was inoculated into 25 cc. of plain bouillon, and this second subculture incubated for 18 to 20 hours. At the end of that time the bacteria were centrifugalized out, washed twice in saline, and an emulsion of these washed organisms in salt solution was used for the agglutination test. In this way, the error that would result from the pres-

ence of the immune serum in which the organisms had been grown was avoided. It will be seen that the last subculture contained serum in dilution of only 1:5,000; after washing twice in saline even this small amount must have been largely removed. Antipneumococcus serum does not agglutinate in a dilution of immune serum higher than 1:500, hence the reaction obtained could not have been due to a small amount of serum adhering to the bacteria. Agglutinations were made with the two types of antipneumococcus horse serum, and with normal horse serum. In each case one tube contained equal parts of the undiluted serum and bacterial emulsion, and the others 0.9 cc. of serum dilution and 0.1 cc. of bacterial emulsion. Tubes were kept in a water bath at 37°C. for 2 hours, and over night on ice, and readings were taken at the end of that time. Complete reaction (++) was recorded when the bacteria were entirely sedimented with marked precipitation, leaving the supernatant fluid clear; almost complete (+±), when the bacteria were sedimented with less marked precipitation; incomplete (+), when marked clumping could be seen but the clumps were still readily shaken from the bottom of the tube; partial (±), when the clumps had not sedimented, but could be distinguished macroscopically as a fine granulation; negative (−), when no clumping was present.

Table I shows the result of three agglutination tests, the organism in each instance having descended from the same mouse passage culture of Pneumococcus Type I. The original culture showed typical agglutination reaction, virulence, and protection by homologous serum. Subculture A was grown successively in Immune Serum I bouillon, Subculture B successively in normal serum bouillon, Subculture C successively in plain bouillon. After 4 immune serum treatments, Subculture A lost intensity of reaction with its homologous serum, from complete to incomplete, and became partially agglutinated with the heterologous serum. After 20 treatments in immune serum the reaction was still incomplete with the homologous serum and had become almost complete with the heterologous serum. After 15 transfers in plain bouillon, subsequent to 22 treatments in immune serum, the organisms had regained somewhat in intensity of reaction with the homologous serum, but still agglutinated partially with the heterologous serum. Subcultures B and C, on the contrary, maintained

TABLE I.

Agglutination Tests of Serum Treated Strain I 114, Pneumococcus Type I, 114 Animal Passages, before Serum Treatment.

Subcultures in each test were grown for two successive transfers in plain bouillon subsequent to serum treatment and a twice washed emulsion of centrifugalized organisms was used. Tube 1 in each case contained 0.3 cc. of bacterial emulsion and 0.3 cc. of undiluted serum; the remaining tubes contained 0.9 cc. of serum dilution and 0.1 cc. of bacterial emulsion. Readings were made after the tubes had been kept in a water bath at 37°C. for 2 hours and over night on ice.

Serum dilutions.	Subculture A treated in Immune Serum I.			Subculture B treated in normal serum.			Subculture C untreated.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain I 114[7]*. 4 serum treatments.									
Nov. 6, 1915.									
Undiluted.	+	±	−	++	−	−	+	−	−
1:10	+	±	−	++	−	−	+	−	−
1:20	+	±	−	++	−	−	++	−	−
1:40	+	−	−	++	−	−	++	−	−
1:50	+	−	−	+	−	−	++	−	−
1:100	+			−			−		
1:200	±			−			−		
1:400	±			−			−		
1:500	−			−			−		
Salt control.	−			−			−		
Strain I 114[23]. 20 serum treatments.									
Nov. 30, 1915.									
Undiluted.	+	+±	−	++	−	−	++	−	−
1:10	+	+±	−	++	−	−	++	−	−
1:20	+	+±	−	++	−	−	++	−	−
1:40	+	+±	−	++	−	−	++	−	−
1:50	+	+±	−	++	−	−	++	−	−
1:100	+			−					
1:200	+			−					
1:400	+			−					
1:500	+			−					
Salt control.	−			−					

TABLE I—*Concluded.*

Serum dilutions.	Subculture A treated in Immune Serum I.			Subculture B treated in normal serum.			Subculture C untreated.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
Strain I 114[38]. 15 transfers in plain bouillon subsequent to serum treatment. Dec. 21, 1915.									
Undiluted.	±	±	−	+	−	−			
1:10	++	±	−	+	−	−			
1:20	+	±	−	+	−	−			
1:40	+	±	−	+	−	−			
1:50	+	±	−	+	−	−			
1:100	+			+					
1:200	+			−					
1:400	−			−					
1:500	−			−					
Salt control.	−			−					

* In all the tables the numerals after the culture indicate the number of mouse passages. The exponent indicates the number of generations removed from the last passage.

throughout the course of treatment the same intensity of reaction with the homologous serum and did not become agglutinated either in the heterologous or in the normal serum.

Table II shows the result of three agglutination tests made with two subcultures, both from the same mouse passage culture of a Pneumococcus Type II, which showed typical agglutination reaction, virulence, and protection before treatment. Subculture A was grown successively in Immune Serum II bouillon, Subculture B successively in normal serum bouillon. After 53 treatments with immune serum, Subculture A was only partially agglutinated in the homologous Serum II, but was completely agglutinated in the heterologous Serum I, and incompletely agglutinated in normal serum. After 75 treatments in immune serum, it was still only partially agglutinated in the homologous serum, completely agglutinated in the heterologous, and, this time, not agglutinated in normal serum. After 47 transfers in plain bouillon subsequent to serum treatment, the partial agglutination with the homologous serum and complete agglutination with the

TABLE II.

Agglutination Tests of Serum Treated Strain II 34, Pneumococcus Type II, 34 Animal Passages, before Serum Treatment.

The same technique was used as in the tests in Table I.

Serum dilutions.	Subculture A treated in Immune Serum II.			Subculture B treated in normal serum.		
	Immune Serum I.	Immune Serum II.	Normal serum.	Immune Serum I.	Immune Serum II.	Normal serum.
	Strain II 34^{68}. 53 serum treatments.					
Oct. 1, 1915.						
Undiluted.	++	±	+	−	++	−
1:10	++	±	+	−	++	−
1:20	++	±	+	−	++	−
1:40	+	±	+	−	++	−
1:50	+	±	+	−	++	−
1:100		±			++	
1:200		±			−	
1:400		−			−	
1:500		−			−	
Salt control.		−			−	
	Strain II 34^{90}. 75 serum treatments.					
Dec. 2, 1915.						
Undiluted.	++	±	−	−	++	−
1:10	++	±	−	−	++	−
1:20	++	±	−	−	++	−
1:40	++	±	−	−	++	−
1:50	++	±	−	−	±	−
1:100	++	±			−	
1:200	−	−			−	
1 400		−			−	
1:500		−			−	
Salt control.		−			−	
	Strain II 34^{116}. 47 transfers in plain bouillon subsequent to serum treatment.					
Dec. 2, 1915.						
Undiluted.	++	±	−	−	++	−
1:10	++	±	−	−	++	−
1:20	++	±	−	−	++	−
1:40	+	±	−	−	++	−
1:50	+	±	−	−	++	−
1:100		±			−	
1:200		−			−	
1:400		−			−	
1:500		−			−	
Salt control.		−			−	

heterologous serum persisted. In this experiment, also, the organisms grown in normal serum were still completely agglutinated with homologous serum and were in no instance agglutinated with the heterologous or normal serum. It is to be noted, however, in spite of the incompleteness of the agglutination of the treated organisms in the homologous serum, that the agglutination titer in homologous serum was higher than that in heterologous serum, and also higher than that of normal untreated strains in homologous serum.

These tests were repeated a number of times with many different strains, always with the same result; the longer a strain was treated with its homologous serum, the less agglutinable it became in that serum, and the more agglutinable in the heterologous serum. On the contrary, the control strains cultured in normal serum and plain bouillon, retained throughout the experiments the typical agglutination with the homologous serum and were never agglutinated by the heterologous or normal serum. It was also noted that the immune serum treated strains sometimes were agglutinated with normal horse serum, but they did not agglutinate spontaneously in salt solution, nor did they agglutinate in other immune sera, such as diphtheria and tetanus antitoxin, anti-influenza serum, nor in an immune sheep serum obtained by the injection of an antigen prepared by the solution of pneumococci in sodium cholate. Bull (16) has tested an immune serum treated strain of Type I and found that it is agglutinated *in vivo* and *in vitro* by normal rabbit serum, while the control strain is not.

Variations in Virulence.

Simultaneous with the change in agglutinability, the immune serum treated cultures showed a pronounced loss of virulence for white mice and rabbits. It often required a million times more of an immune serum treated culture to kill than of the control normal serum treated culture. Virulence tests were always made with cultures at least twice removed from the serum treated culture in which 1 cc. contained only $\frac{1}{5,000}$ part of immune serum. As 0.1 cc. of this serum is required to protect against 0.1 cc. of a normal strain, this loss of virulence cannot be attributed to the protective value of the serum present. In rabbits it was found

that the washed bacteria from 15 cc. of a bouillon culture of treated organisms would not kill, while 0.000005 cc. of culture of untreated organisms killed in 24 hours. In white mice it often required the bacteria from 5 cc. of a culture of treated organisms to kill, while 0.000001 cc. of culture of untreated organisms killed in 24 hours.

Table III shows the comparative virulence of the immune and normal serum treated cultures of Pneumococcus Type I and Pneumococcus Type II, the agglutination tests of which are given in Tables I and II. The virulence of Subculture I 114 B, treated 12 times with normal serum was such that 0.000001 cc. of a 20 hour bouillon culture killed in 56 hours, while the fatal dose of Subculture A, treated 12 times with Immune Serum I, was 0.5 cc. of a 20 hour bouillon culture. After 20 treatments with immune serum, 1 cc. of a broth culture of Subculture A did not kill, and after 46 treatments the

TABLE III.

Virulence of Serum Treated Strains of Pneumococci.

The tests were made by intraperitoneal injection into white mice. All serum cultures were transferred at least twice before injection into such amounts of bouillon that the dilution of serum in the culture used was at least 1: 5,000. Autopsies were performed on all animals and smears were made from the peritoneal exudate. Pneumococci were found in all cases.

Test I.

Virulence, Pneumococcus Type I.

Amount of culture injected.	12 serum treatments.		20 serum treatments.		46 serum treatments.	
	Treated in Immune Serum I.	Treated in normal serum.	Treated in Immune Serum I.	Treated in normal serum.	Treated in Immune Serum I.	Treated in normal serum.
cc.						
5.0	—	—	—	—	D. 18 hrs.	—
1.0	—	—	S.*	—	S.	—
0.5	D. 24 hrs.	—	S.	—	S.	—
0.1	S.	—	S.	—	—	—
0.01	S.	—	S.	—	—	—
0.001	S.	—	S.	—	—	—
0.0001	S.	D. 18 hrs.	S.	D. 26 hrs.	—	D. 36 hrs.
0.00001	D. 24 hrs.†	" 56 "	—	" 25 "	—	" 36 "
0.000001	—	" 56 "	—	" 25 "	—	" 36 "

TABLE III—*Concluded.*

Test II.

Virulence Tests of Serum Treated Pneumococcus Type II Made with Cultures Which Had Received 27 and 61 Transfers in Plain Bouillon Subsequent to 55 Serum Treatments.

Virulence, Pneumococcus Type II.

Amount of culture injected.	27 transfers in plain bouillon.		61 transfers in plain bouillon.	
	Treated in Immune Serum II.	Treated in normal serum.	Treated in Immune Serum II.	Treated in normal serum.
cc.				
2.0	D. 18 hrs.	—	—	—
1.5	—	—	S.	—
1.0	S.	—	S.	—
0.5	D. 36 hrs.	D. 18 hrs.	S.	—
0.1	S.	" 18 "	S.	—
0.01	S.	" 36 "	—	—
0.001	S.	" 24 "	—	—
0.0001	—	" 24 "	—	D. 23 hrs.
0.00001	—	" 20 "	—	" 48 "
0.000001	—	—	—	" 48 "

* In the tables D. stands for died; S., for survived. The figures represent the number of hours after injection before the death of the animal.

† No pneumococci were found in the heart's blood of this animal.

animal receiving the bacteria from 1 cc. of a broth culture of Subculture A survived. Subculture B, however, which was treated an equal number of times with normal serum, maintained a high virulence, 0.000001 cc. of a broth culture killing regularly.

In Test II, Table III, is shown the comparative virulence of an immune and normal serum treated culture of Pneumococcus Type II 34. Each culture had received 55 serum treatments and had been passed subsequently through a large series of broth transfers. After 27 transfers in plain broth the fatal dose of the immune serum treated culture was 0.5 cc., or more, while 0.00001 cc. of the normal serum treated strain killed a mouse in 20 hours. After 61 transfers in plain bouillon 1.5 cc. of the immune serum treated culture failed to cause death, although 0.000001 cc. of the normal serum treated culture was fatal to a mouse in 48 hours.

It will be seen from these tables that the longer the pneumococcus is grown in immune serum the less virulent it becomes, while the same strain grown in normal serum retains its original virulence even after long periods of growth in plain bouillon. The immune serum treated organisms retain the avirulent characteristics after as many as 75 transfers in plain bouillon subsequent to serum treatment. This fact demonstrates conclusively that the loss of virulence is not due to the protective action of the immune serum present.

A study of the capsule formation in the immune and normal serum treated cultures demonstrated the interesting fact that while the latter upon injection into mice formed capsules easily demonstrable by the Hiss method, the immune serum treated organisms showed no demonstrable capsules under similar conditions. It is possible that the loss of virulence noted may in some way be related to the apparent absence of capsule formation.

Variations in Phagocytability.

The phagocytability of the organisms grown in immune serum and of those grown in normal serum was tested with guinea pig leukocytes in the presence of normal and immune horse serum by the Neufeld method. The organisms grown in normal serum were phagocyted only in the presence of immune serum. Those grown in immune serum after at least two subsequent broth passages were phagocyted in the presence of both immune and normal horse serum. The organisms grown in immune serum were also phagocyted *in vivo* in the normal rabbit, a reaction which does not take place with the normal virulent pneumococcus. Growth in immune serum has, therefore, made susceptible to the phagocytic action of guinea pig leukocytes a culture of pneumococcus previously resistant to such action. This phenomenon may also be related to the loss of virulence which occurs as the result of growth in immune serum, since it is known that non-virulent pneumococci are more readily phagocyted than highly virulent strains.

Variations in Antigenic Properties.

In order to test the possibility of variation in the antigenic properties of immune serum treated strains, two antigens were prepared in the following manner. Emulsions of washed bacteria, killed by heating at 56°C. for 45 minutes, were prepared from equal amounts of two bouillon cultures of Pneumococcus Type II 34, one of which had been treated for 60 successive transfers with Serum II, the other for the same number of transfers with normal horse serum. Immune sera were prepared from these antigens by intravenous injection of rabbits. The immunization of each animal was carried out in a corresponding manner, and the serum obtained for the tests at comparable intervals of time. The immune rabbit sera thus prepared were tested for their agglutination reaction with normal and immune serum treated pneumococci of both Types I and II.

Examination of Table IV shows that the immune response as measured by agglutinins is slower in the rabbits immunized with immune serum treated pneumococci than in those immunized with the normal serum treated organisms. It is further evident that the serum of rabbits immunized to immune serum treated Pneumococcus Type II contains agglutinins for the immune serum treated organisms of both Types I and II, but does not possess antibodies for the strains treated

TABLE IV.

Agglutination Reaction of Sera from Rabbits Immunized to Normal and Immune Serum Treated Strains of Pneumococcus Type II.

Agglutination Reaction of Sera Obtained 4 Weeks after the Beginning of Immunization.

Culture used for agglutination.	Serum from rabbits immunized with immune serum treated Pneumococcus Type II.		Serum from rabbits immunized with normal serum treated Pneumococcus Type II.	
	Rabbit 29C	Rabbit 30C	Rabbit 31C	Rabbit 32C
Normal Pneumococcus Type II	−	−	++	++
Immune serum treated Pneumococcus Type II	−	−	±	±

TABLE IV—*Concluded.*

Agglutination Reaction of Sera Obtained 6 Weeks after the Beginning of Immunization, Including Cross Agglutination Reactions with Normal and Immune Serum Treated Pneumococci Types I and II.

Dilutions of serum.	Serum from rabbit immunized with immune serum treated Pneumococcus Type II.				Serum from rabbit immunized with normal serum treated Pneumococcus Type II.			
	Normal Serum Treated Culture II.	Immune Serum Treated Culture II.	Normal Serum Treated Culture I.	Immune Serum Treated Culture I.	Normal Serum Treated Culture II.	Immune Serum Treated Culture II.	Normal Serum Treated Culture I.	Immune Serum Treated Culture I.
Undiluted.	−	+	−	+	++	+	−	+
1:10	−	+	−	+	++	+	−	+
1:20	−	+	−	+	++	+	−	+
1:40	−	+			+	+		
1:50		+			+	+		
1:100		+			+	+		
1:200		+			−	+		
1:400		−			−	−		

with normal serum. On the other hand, the sera of rabbits immunized to a Type II pneumococcus which had been grown in normal serum for a similar number of transfers, agglutinated not only the homologous normal strain, but reacted, although less sharply, with immune serum treated pneumococci of both Types I and II.

Variations in Absorption Properties.

It has been demonstrated by Avery (17) that absorption of Antipneumococcus Serum I or II with the homologous pneumococcus removes from the serum all agglutinins for the homologous organism, while saturation of Serum I with a Type II organism does not remove the agglutinins present for Type I, and the same result is obtained when an attempt is made to exhaust the antibodies of Serum II with Pneumococcus Type I. Saturation of the Antipneumococcus Serum I or II with homologous immune serum treated organisms, removes all agglutinins for both homologous and heterologous immune serum treated pneumococci, but does not remove the agglutinins for the homologous normal serum treated organisms. It is also found that saturation of antipneumococcus serum with

the homologous type of normal serum treated organisms removes the agglutinins not only for these pneumococci, but for all immune serum treated strains.

Comparative Absorption of Antipneumococcus Serum with Immune Serum Treated and Normal Serum Treated Pneumococci.

Technique.—To 5 cc. of Antipneumococcus Serum II was added the washed bacterial residue from 25 cc. of a 20 hour bouillon culture of Pneumococcus Type II which had been treated for 65 transfers with Immune Serum II. The mixture was kept at 37°C. for 30 minutes and for 18 hours at ice temperature. A similar mixture was prepared by adding to the same amount of Serum II the bacterial residue from 25 cc. of a 20 hour bouillon culture of a strain of Pneumococcus Type II which had been treated for 65 transfers with normal horse serum, and this mixture was also kept at 37°C. for 30 minutes and for 18 hours at ice temperature. At the end of that time both mixtures were centrifugalized and the clear supernatant fluids again absorbed with fresh bacterial residues of the immune and normal serum treated cultures. Agglutination tests were then made with these absorbed sera.

TABLE V.

Agglutination of Antipneumococcus Serum II before and after Absorption with Normal and Immune Serum Treated Strains of Pneumococcus Type II.

Strains used for agglutination.	Before absorption.	After absorption with normal serum treated Type II.	After absorption with immune serum treated Type II.
Pneumococcus Type II, normal serum treated	++	−	++
Pneumococcus Type II, immune serum treated	++	−	−
Pneumococcus Type I, normal serum treated	−	−	−
Pneumococcus Type I, immune serum treated	+	−	−

Table V gives the result of an absorption experiment with Antipneumococcus Serum II. Absorption was repeated until the normal serum treated organisms had absorbed from the serum all the agglutinins for this strain. This serum then had lost, also, the agglu-

tinins for the immune serum treated organisms which it formerly contained, while the serum which had been absorbed with equal amounts of the immune serum treated organism, although it had lost the agglutinins for the homologous immune serum treated pneumococcus, still retained an apparently undiminished quantity for the homologous normal serum treated organism.

Reversion to Type of the Altered Strains on Animal Passage.

Attempts were made to ascertain whether these variations in agglutinability, virulence, and capsule formation would persist after repeated animal passage. This study revealed the fact that all strains so far tested reverted to the original type by passage through the animal body, the number of passages required for this reversion depending upon the number of treatments in immune serum which the organism had received. Strains which had received from 6 to 12 treatments regained virulence and specific agglutinability upon 1 animal passage, while it required 3 or more successive passages to restore the virulence and agglutinability of strains which had received from 50 to 100 serum treatments.

TABLE VI.

Effect of Animal Passage upon Agglutination and Virulence of Serum Treated Pneumococci.

Test I.

Agglutination and Virulence Tests Made before and after Animal Passage of Culture I 112[9], Which Had Received 6 Serum Treatments and 2 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after animal passage.	Result before animal passage.	Result after animal passage.
	Virulence of organisms treated in Immune Serum I.		Virulence of organisms treated in normal serum.	
cc.				
1.0	D. 16 hrs.	D. 18 hrs.	D. 16 hrs.	—
0.2	" 40 "	" 18 "	" 16 "	—
0.1	" 40 "	" 18 "	" 16 "	D. 18 hrs.
0.01	S.	" 18 "	" 20 "	" 18 "
0.001	S.	" 40 "	" 24 "	" 20 "
0.0001	S.	" 22 "	" 40 "	" 20 "

TABLE VI—*Continued.*

Agglutination of Culture I 112[9], before and after Animal Passage.

Serum used for agglutination.	Culture treated in Immune Serum I.		Culture treated in normal serum.	
	Result before animal passage.	Result after animal passage.	Result before animal passage.	Result after animal passage.
Immune Serum I	+	++	++	++
" " II	±	−	−	−
Normal horse serum	++	−	−	−

Test II.

Agglutination and Virulence Tests Made before and after Successive Animal Passages of Culture I 114[62], Which Had Received 59 Serum Treatments and Subsequently 2 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after first animal passage.	Result after second animal passage.
Virulence of organisms treated in Immune Serum I.			
Bacteria from 10 cc. culture	D. 16 hrs.	−	
" " 5 " "	" 16 "	−	
2.0 cc. culture	" 16 "	D. 16 hrs.	D. 36 hrs.
1.5 " "	S.	S.	−
1.0 " "	S.	S.	S.
0.5 " "	S.	−	−
0.1 " "	−	S.	−
0.001 " "	−	−	S.
Agglutination in Serum I	+	+	++
" " " II	±	±	−
" " normal serum	±	+	−
Virulence of organisms treated in normal serum.			
0.01 cc. culture	D. 36 hrs.	−	
0.001 " "	" 20 "	−	
0.00001 " "	" 25 "	D. 22 hrs.	
0.000001 " "	" 36 "	" 21 "	
Agglutination in Serum I	++	++	
" " " II	−	−	
" " normal serum	−	−	

TABLE VI—*Concluded.*

Test III.

Agglutination and Virulence Tests Made before and after Successive Animal Passages of Culture II 34[124], Which Had Received 109 Serum Treatments and Subsequently 4 Passages in Plain Bouillon before Testing.

Amount of culture injected.	Result before animal passage.	Result after first animal passage.	Result after second animal passage.	Result after third animal passage.
Organisms treated in Immune Serum II.				
Bacteria from 10 cc. culture....	D. 36 hrs.	−	−	−
" " 5 " "	S.	−	−	−
2.0 cc. culture	−	D. 18 hrs.	−	−
1.0 " "	S.	" 18 "	−	−
0.5 " "	−	" 18 "	−	−
0.1 " "	−	" 18 "	D. 16 hrs.	−
0.001 " "	−	" 18 "	−	D. 36 hrs.
0.0001 " "	−	" 36 "	D. 16 hrs.	" 36 "
0.00001 " "	−	−	" 20 "	" 36 "
0.000001 " "	−	−	" 24 "	" 36 "
Agglutination in Serum I.....	+	+	+	−
" " " II.....	++	++	++	++
" " normal serum.	+	−	−	−
Organisms treated in normal serum.				
0.01 cc. culture.....	D. 16 hrs.	−		
0.001 " "	" 16 "	−		
0.00001 " "	" 20 "	D. 24 hrs.		
0.000001 " "	" 22 "	" 36 "		
Agglutination in Serum I.....	−	−		
" " " II.....	++	++		
" " normal serum.	−	−		

Table VI shows the result of animal passage of three different strains of immune and normal serum treated pneumococci. Test I shows the result of 1 animal passage of a Pneumococcus Type I which had received 6 serum treatments, original virulence and typical agglutinability being regained upon 1 animal passage. Before passage it required 0.1 cc. of culture of the immune serum treated organism to kill mice in 40 hours, and the organisms were agglutinated with Serum II and normal serum; after passage 0.0001 cc. of culture killed

in 22 hours and the bacteria were agglutinated only in Serum I. Test II shows the result of 3 animal passages of a Pneumococcus Type I which had received 59 serum treatments. In this case it required 3 animal passages to cause the strain to revert to original agglutinability, and the virulence had not been materially raised by this number of passages. Test III shows the result of 4 animal passages of a Pneumococcus Type II which had received 109 serum treatments. With this strain 4 passages were required to restore the original agglutinability, although the virulence was raised by 1 passage. In all cases control tests were made with a normal serum treated organism, which had neither lost in virulence nor in specific agglutinability. A great number of similar tests have demonstrated that these immune serum treated strains are readily caused to revert to the original type by animal passage.

DISCUSSION.

The effect of the treatment of pneumococci with immune serum may be attributed to a suppression of certain receptors, similar to the phenomenon observed by Cole with inagglutinable typhoid strains, since the serum treated pneumococci become less specifically agglutinable, can no longer absorb the agglutinins for the normal strains, and, when injected into the animal body, do not produce agglutinating sera for the normal strains.

The effect produced on the virulence of the pneumococcus by immune serum treatment is not identical with that produced in other bacteria by treatment with immune sera. The pneumococcus, instead of becoming more virulent, (serum-fast or immune) with immune serum treatment, becomes much less virulent. This loss of virulence is one of the most noticeable effects of the treatment with the immune serum and, as mentioned before, cannot be explained by the protective action of the serum present, since it persists after 60 or 70 transfers in plain bouillon subsequent to serum treatment, when the amount of serum present in the culture is too small to compute, and after the organisms have passed through so many generations that there could surely be no immune substances from the serum still adherent to them. It would seem that some biologic change must have

taken place in the organism, transforming it from the virulent to the avirulent type. This inference is confirmed by the fact that these strains rendered avirulent have lost capsules and are phagocyted in normal serum.

At present we have no explanation to offer in regard to this difference between the virulence of the typhoid bacillus and the pneumococcus when grown in their immune sera. Since antityphoid serum is bactericidal and reacts best in the presence of complement, and since antipneumococcus serum possesses no demonstrable bactericidal properties and its agglutinating and protective action is not increased by the addition of complement, it is possible that the different results of serum treatment upon these bacteria with such widely separated biologic characteristics may, in some way, be attributable to the differences in the properties of these two sera.

It is interesting to note that the variations in pneumococci produced by treatment with immune serum do not persist after animal passage. Very few passages through the animal body cause the changed organism to revert to the original type with characteristic agglutinability, virulence, etc.

CONCLUSIONS.

1. The growth of virulent pneumococci in homologous immune serum produces (*a*) variations in agglutinability, (*b*) decrease in virulence, (*c*) inhibition of capsule formation, (*d*) increase in phagocytability with normal serum, and (*e*) change in absorption power and antigenic properties.

2. Reversion to the original type in these changed forms takes place upon animal passage.

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THE CONDITIONS AND CHARACTERS OF THE IMMUNITY PRODUCED IN THE GUINEA PIG BY INSTILLATION OF HORSE SERUM INTO THE NOSE.

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In a recent communication[1] we showed in detail that when a few drops of horse serum are instilled, on two or more occasions, into the nostrils of a guinea pig the animal may become affected in radically different ways. An intravenous injection of the antigen given 16 days after the last instillation may lead to more or less profound shock or speedy anaphylactic death. But in a certain proportion of cases the toxic injection produces no obvious reaction. It would appear that the animal had not absorbed the serum introduced into the nose were it not that a second toxic injection, given 24 days after the first, may also be withstood. Therefore, we must conclude that the guinea pig was made primarily refractory, not sensitive to the dose of horse serum introduced into the vein. It seemed to us important to determine the experimental conditions according to which a series of nasal instillations of serum would, on the one hand, render the guinea pig hypersusceptible, or, on the other, insusceptible to a toxic dose of the serum. Our work last year was based on the hypothesis that the biological results of the nasal treatment depended on the time intervals between successive instillations. But, although it became plain that the rhythm of dosages by the nose was not a matter of indifference, we were unable to explain by it the variable effects of the toxic injection.

A more favorable issue has attended the present series of experiments which was suggested through an occurrence which implied a definite relation between the amount of antigen and its qualitative

[1] Sewall, H., and Powell, C., *Arch. Int. Med.*, 1915, xvi, 605.

effects. Immediately, therefore, protocols were prepared for the investigation of the influence of the quantity of serum introduced into the nose upon the specific reactivity of the guinea pig.

The Qualitative Effects of Serum Introduced into the Nose Depend upon the Quantity Instilled.

Our experiments were performed upon young animals, averaging at the outset about 300 gm. in weight. The undiluted Cutter's horse serum was dropped into the nostrils of the animals in the manner previously described.[1] Each drop represented approximately 0.02 cc. The first intravenous or toxic injection, of 0.38 cc., was uniformly given 16 days after the last instillation; the second intravenous injection usually followed the first in 14 days.

Some excerpts from our general results are collected in Table I. The animals in each group were simultaneously carried through

TABLE I.

The Influence of the Quantity of Serum Instilled on the Reaction to Toxic Injections.

Group of guinea pig.	No. in each group.	Amount of serum used in each instillation.	Total no. of instillations.	Intervals between instillations.	No. surviving 1st intravenous injection of 0.38 cc. serum.	No. surviving 2nd intravenous injection of 0.38 cc. serum.
		cc.		*days*		
I	3	0.2	4*	1	0	
II	4	0.2	6†	2	0	
III	2	0.2	4	2	1‡	1
IV	6	0.1	6	2	4	2
V	2	0.04	4	2	2	2
VI	6	0.04	6	2	6	3
VII	6	0.04	12	1	5	3
VIII	6	0.02	12	1	6	4

* A fifth instillation of 0.2 cc. was given 14 days after the fourth.

† A seventh instillation was given 14 days after the sixth and the toxic injection delayed correspondingly.

‡ The amount of serum in the first intravenous injection was reduced to 0.25 cc.

identical procedures. It will be observed that four separate instillations of serum are sufficient to provoke a maximal biologic response. Of nine guinea pigs receiving instillations of 0.2 cc. in quantity (Groups I, II, and III), only one survived the first toxic injection and in this case the amount of serum given by the vein was reduced to 0.25 cc. From the reaction manifested by the animal it is probable that it also would have succumbed to the usual injection of 0.38 cc.

Of twenty guinea pigs receiving instillations of 0.04 cc. or less only one succumbed to the first intravenous injection of 0.38 cc. of horse serum. Twelve of these twenty animals withstood a second intravenous injection of 0.38 cc. of serum 14 days or more after the first. There can be no doubt, therefore, that three-fifths of the animals were strongly immunized by the preliminary instillations of serum. With regard to the seven guinea pigs which succumbed to the second toxic injection the question arises: Did they completely fail to absorb the serum instilled and thus become sensitized by the first toxic injection in the ordinary way, or was the protection conferred by the instillations insufficient in degree to balance the shock of the large second injection? The following considerations led us to adopt the latter explanation.

Many observations have impressed us with the conclusion that the fatality attending the second toxic injection is roughly proportionate to the degree of anaphylactic reaction manifested with the first.

In reviewing our notes we find that of 81 guinea pigs in which record was made of the degree of reaction manifested to intravenous injections following a preliminary course of nasal instillations, in eighteen cases little or no definite shock was caused by the first intravenous injection; of these only three animals succumbed to the second injection of 0.38 cc. of serum, a mortality of less than 17 per cent. Of thirty-nine animals surviving the first intravenous injection after greater or less shock no less than twelve succumbed to the second injection, a mortality of more than 30 per cent.

Another reason for believing that some degree of absorption attends the application of minute quantities of foreign serum to the mucous membrane of the nose consists in the fact that invariably a secretion of saliva follows within a few seconds the fall of a single drop of serum into the nostril; when the lower lip of the animal is

depressed saliva is seen to accumulate about the incisor teeth, provided it has not been swallowed as formed. This has been assumed by us to indicate absorption.

If the death of our animals with the second toxic injection is due, as we suppose, not to inertness of the nasal instillations of serum but to the insufficiency of their immunizing power, it should be easy to demonstrate a weak protective power in the instillations by reducing the amount of the second toxic injection. This we have done to some extent and have found that when the quantity of serum in the second toxic injection is reduced one-half the animals usually survive. There still remains, however, a certain proportion of animals in which the grade of immunity is still much too low to resist this amount of antigen. It is an impression gained from many experiences, but not especially investigated, that the mortality from the second intravenous injection is higher, other things remaining the same, when the second injection follows the first after an interval of 14 days rather than 24 days, the interval formerly employed by us.

The Amount of Serum Absorbed and Its Biologic Effect Depend upon the Method of Instillation.

In all our experiments the complex living mechanisms responded exactly, as depicted in Table I, to the crude method we employed to introduce the serum into the body. In our confirmatory experiments a series of irregularities fortunately developed, which, when investigated, led to a better understanding of the conditions of biologic response. Thus, in a group of six guinea pigs instilled six times on alternate days with 0.2 cc. of serum, it was expected that all would succumb to the toxic injection of 0.38 cc. given 16 days after the last instillation. On the contrary, three of the animals survived, one after very severe shock and two with little or none. The second intravenous injection was reduced to 0.19 cc., and given 30 days later. The animal which had been previously shocked died, the other two easily survived.

Again six guinea pigs were instilled as above, but the quantity of serum used at each instillation was reduced to 0.04 cc. It was expected that all would survive the first intravenous injection of 0.38

cc. of serum. On the contrary, four animals succumbed to it. It was then realized that the method of instilling the serum had been radically different in the two sets of experiments. In the first case the procedure of instillation had been hurried; in the second it was prolonged and attended by obvious vital reactions, such as abundant salivary secretion. It was necessary to determine whether our results could be definitely modified by varying the area of contact between the serum and mucous membrane.

When the head of the animal receiving the instillation is held with its long axis in a vertical plane the serum is probably confined to the respiratory canal and does not reach the turbinate mucous membrane. But if, while the guinea pig is held in the supine position, the head is well extended, the opportunity is given for the serum to gravitate through the complex turbinate convolutions, and this to an extent dependent on the time during which the posture is maintained.

Accordingly, two groups of four guinea pigs each were prepared to test this reasoning. All the animals received five instillations of 0.1 cc. of serum on alternate days. But in the first group the heads of the animals were held vertically and the serum was dropped quickly, from 15 to 30 seconds being consumed in administering the five drops of each dose. In the second group of animals the heads were held well extended and from 3 to 5 minutes were occupied in each instillation. The first toxic injection of 0.38 cc. of serum was given after the usual interval. All four guinea pigs of the first group easily survived; three of the second group died and the remaining one was strongly shocked. We therefore conclude that the biologic effect of a given dose of serum depends chiefly upon the extent of its contact with the mucous membrane of the turbinate apparatus.

It is probable that several other factors, including quality and temperature of the horse serum, take part in determining the coefficient of absorption.

As will be shown in the following section, great individual differences may distinguish animals in their reaction to the same treatment. The susceptibility to anaphylaxis of different families of guinea pigs is noteworthy. Thus, twelve belonging to a wholly different stock from that from which most of our animals were obtained

during the present year proved peculiarly sensitive to serum treatment. Three of them instilled four times with 0.2 cc. of serum on alternate days succumbed to the usual toxic injection, as was to have been expected. The other nine animals received like treatment but with only 0.04 cc. of serum in each instillation. The first intravenous injection of 0.38 cc. of serum killed one and shocked the remainder to a considerably greater degree than usual under these conditions.

Gradations of Sensibility Induced by Serum Instillations.

The reaction to an antigen exhibited by an immunized animal is expressed by a ratio one factor of which is the vital resistance of the host and the other the amount and virulence of the antigen.

In terms of physiology, the plane of immunity is determined by the threshold of irritability to the antigen. This has recently been well brought out by Webb[2] who has successfully inoculated guinea pigs with many thousands of tubercle bacilli of which the minimal lethal dose was 125; but he always failed to establish immunity against recent cultures of which the m.l.d. was only ten bacilli. When a guinea pig is submitted to a series of sensitizing instillations of horse serum it sometimes happens, in possibly 5 per cent of the cases, that with the terminal treatment 8 or 10 days following the first, the animal develops a pronounced attack of asthma with loud, moist bronchial râles.

This is a sign of intense sensitization; we have found the hypersensitiveness to persist at least 60 days and such animals invariably succumb to the first toxic injection as used by us. After three or four sensitizing instillations, another of the same kind repeated after the lapse of 2 weeks produces a greater or less respiratory disturbance in the majority of cases. Such animals succumb to the toxic injection, as do also some which have shown no asthmatic symptoms. Several of our animals which after courses of nasal instillation had been strongly immunized by intravenous injections of serum, when given a nasal instillation of serum after the lapse of some months, showed no reaction whatever. Formerly we were of the opinion that the expression of local sensitization as manifested by asthma required a defi-

[2] Webb, G. B., *J. Lab. and Clin. Med.*, 1916, i, 414.

nite incubation period of 8 or 10 days between the first nasal instillation and that which could produce asthmatic symptoms. Recently, however, we have found that in guinea pigs born of treated mothers and highly sensitized by the subcutaneous injection of 0.1 cc. of horse serum more than 3 months before, well marked asthma could be aroused by the first instillation of serum into the nose. The subject achieves peculiar practical importance in view of the analogous clinical asthma in human beings. Our demonstration of the variability of symptoms of local sensitization of the respiratory apparatus on a constant background of general sensitization makes it questionable as to how reliable tests performed on other peripheral mechanisms, such as the skin, may be for indicating the immunological state of the body as a whole.

The Earlier Instillations of a Series Determine the Biologic Effect of the Whole.

Such results as those depicted in Table I have led us to differentiate our dosages of serum into those which are protective and those which are sensitizing. Previous observations had led to the impression that the two biologic states could be developed one from the other by appropriate intranasal treatment. The following experiments indicate that such is not the case.

To four young guinea pigs were given by the nose six instillations of 0.04 cc. of serum on alternate days. This had been found to be a protective or immunizing treatment. In two of the animals the instillations were continued for four doses but the amount of the serum instilled was increased to 0.2 cc. The remaining two guinea pigs were allowed to rest 16 days and then were likewise given four nasal instillations of 0.2 cc. of serum. Both groups were given intravenous injections of serum 16 days after the last instillations and all the animals survived, with slight symptoms in some cases. A second intravenous injection of 0.38 cc. was likewise survived by all the animals except one of the first group.

In our previous work[1] we had found that as few as two instillations of serum were capable of rendering animals either fatally sensitive to a toxic injection or of inducing an immunity through which

they were able to resist a series of intravenous doses. In Table I several instances are given in which the biologic attitude is determined by a series of four instillations. This seems to us to be a matter of great practical importance and it will be expanded in the final discussion.

Inertness of Serum Administered by the Mouth.

An inconclusive but suggestive experiment was performed upon two guinea pigs by administering four instillations of serum orally. The serum, to the amount of 0.2 cc., was dropped under the tongue on alternate days. The animals showed no response whatever to the first toxic injection and died with the second. The conclusion is that the serum had not been absorbed from the mouth or had been so greatly diluted with saliva as to be ineffective, and that the animals became sensitized by the first injection.

The Biologic Effects of Serum Instilled into the Nose Are of Temporary Duration.

It is generally admitted that guinea pigs sensitized by subcutaneous or other parenteral avenues of injection retain their sensitiveness throughout life. In a former course of experiments we demonstrated fatal anaphylaxis in two guinea pigs that had received the last of a series of nasal instillations of serum 40 days before. But recent observations have indicated to us that immunizing phenomena established through the mucous membrane of the nose are of temporary duration. Two groups were treated, one with a series of six protective instillations of 0.04 cc. and the other with sensitizing doses of 0.2 cc. of serum. 51 days later intravenous injections of 0.38 cc. of serum were given to one animal of the former and two of the latter group. Slight disturbance was manifested by the last two animals, and all three animals died with the second toxic injection administered 15 days later.

Two guinea pigs were given a series of six protective instillations of 0.04 cc. of serum on alternate days. 93 days later each animal received an intravenous injection of 0.38 cc. of serum without response. 14 days later one was given a second intravenous injection

of 0.38 cc. and died. The other animal was given only 0.25 cc. and lived after a moderate reaction.

Two guinea pigs received six sensitizing instillations of 0.2 cc. on alternate days. The first intravenous injection of 0.38 cc. of serum given 51 days later was borne with slight response; a similar injection repeated in 15 days killed both animals.

Three guinea pigs were prepared by six instillations of 0.2 cc. of horse serum. One of these developed an attack of asthma with the last instillation. 60 days later a toxic injection of 0.38 cc. of serum killed the asthmatic guinea pig but produced slight response in the other two. 15 days later one of the remaining animals was given by the vein 0.19 cc. and succumbed after some resistance, the other was given 0.12 cc. of serum and survived with slight response.

Our conclusion is that the immunizing phenomena set up by unsupported nasal instillations of serum gradually disappear within about 3 months, the hypersensitiveness from large instillations first changing to a measure of resistance.

As will be seen later, when relatively large injections of serum are given by the vein to animals within the period of protection afforded by appropriate nasal instillations, the immunity produced is apparently permanent and intensified with time.

In Guinea Pigs Immunized after Preliminary Nasal Instillations the Immunity Is Strengthened with Lapse of Time.

In the paper referred to above we quoted the conclusions of Gay and Southard,[3] to the effect that artificial immunization produced by the injection of serum is but a condition of temporary refractoriness, full sensitiveness returning to the animals if they are kept long enough. How long this period must be is not stated, but our results with guinea pigs immunized through the nose and later treated with a series of intravenous injections of serum led to radically different conclusions. We showed reason to believe that when guinea pigs so handled were brought to resist easily the intravenous injection of a certain amount of serum, say 0.38 cc., with the lapse of time, at least up to 101 days, there was such an increase of refractoriness that

[3] Gay, F. P., and Southard, E. E., *J. Med. Research*, 1908, xviii, 407.

more than 1.0 cc. of serum given intravenously could be equally well tolerated.

We have confirmed our previous findings in the present investigation and at the same time studied a small number of controls immunized through the peritoneal cavity.

Table II gives the histories of fifteen guinea pigs, the last three of which are borrowed from the records of former work. Each of these animals had been prepared by a course of nasal instillations of horse serum, followed at stated intervals by intravenous injections of the same. In each column is recorded the number of days elapsing between the successive injections, and the whole time intervening between the last nasal instillation and the last intravenous injection is represented by the sum of these intervals, *e.g.*, in the case of Guinea Pig 4, this is 102 days. The amount of serum used at each injection is also noted, and the signs L_0 to L+++++indicate varying degrees of anaphylactic reaction, while D shows that death followed the dose given. Deferring consideration of Guinea Pigs 10, 11, and 12, we see that after a certain degree of resistance had been established, the tolerance of the animals against serum injected into the vein apparently progressively increased with the lapse of time without treatment. In Animals 1 to 3, inclusive, the nasal instillations of 0.2 cc. of serum plainly caused sensitization so that repeated small intravenous injections were necessary to establish a fair degree of tolerance. This is especially noticeable in Guinea Pig 3, which was one of a group of four animals similarly prepared, the others having succumbed early to small injections. Even so it is seen that an extraordinary increase of resistance develops with time after the fourth intravenous injection. It is obvious that there is some metabolic strain towards an equilibrium of increased resistance against disturbance by the antigen. A comparison of these cases with those of Table III, in which the preparation was by relatively large intraperitoneal injections of serum, shows that in the latter animals what we call the metabolic strain is towards an equilibrium of hypersensitiveness.

Guinea Pigs 4 to 15 of Table II were prepared by small nasal instillations such as we have found to confer primary protection. It is seen that the rule is that such a course of instillations reinforced by two intravenous injections is sufficient to establish a ten-

TABLE II.

The Influence of Time on the Development of Immunity in Guinea Pigs Prepared by Nasal Instillation and Tested by the Intravenous Injection of Horse Serum.

No. of guinea pig.	Preliminary treatment.	First intravenous injection. 1. Amount of serum. 2. No. of days since last instillation. 3. Result.	1. Amount of serum. 2. No. of days since last injection. 3. Result.				
			Second intravenous injection.	Third intravenous injection.	Fourth intravenous injection.	Fifth intravenous injection.	Sixth intravenous injection.
1	Six instillations of 0.2 cc. serum on alternate days.	0.06 cc. 13 L++	0.06 cc. 23 L_0	0.19 cc. 16 L+	0.19 cc. 19 L	0.75 cc. 92 L++	0.75 cc. 114 L+
2	Same as No. 1.	0.06 cc. 13 L++	0.13 cc. 23 L+	0.25 cc. 16 L++	0.25 cc. 19 L+	0.75 cc. 92 L++	
3	Five instillations of 0.2 cc. at intervals of 1 to 4 days. (Very sensitive.)	0.02 cc. 15 L++	0.05 cc. 21 L++	0.13 cc. 16 L+++	0.13 cc. 19 L+	0.5 cc. 96 L++	0.75 cc. 125 L+
4	Six instillations of 0.1 cc. on alternate days.	0.38 cc. 16 L	0.38 cc. 16 L++++	0.75 cc. 70 L			
5	Six instillations of 0.04 cc. on alternate days.	0.38 cc. 16 L_0	0.38 cc. 16 L+	0.88 cc. 69 L+			
6	Same as No. 5.	0.38 cc. 16 L+	0.38 cc. 16 L++	0.81 cc. 69 D			
7	Same as No. 5.	0.36 cc. 16 L_0	0.38 cc. 16 L++	0.75 cc. 67 L++			
8	Six instillations of 0.04 cc. followed by four of 0.2 cc. on alternate days.	0.31 cc. 16 L+	0.38 cc. 14 D				

TABLE II.—*Concluded.*

No. of guinea pig.	Preliminary treatment.	First intravenous injection. 1. Amount of serum. 2. No. of days since last instillation. 3. Result.	1. Amount of serum. 2. No. of days since last injection. 3. Result. Second intravenous injection.	Third intravenous injection.	Fourth intravenous injection.	Fifth intravenous injection.	Sixth intravenous injection.
9	Same as No. 8.	0.38 cc. 16 L	0.5 cc. 68 L+	0.75 cc. 122 L+++			
10	Twelve instillations of 0.02 cc. daily.	0.31 cc. 17 L	0.38 cc. 16 L++	0.75 cc. 64 D			
11	Same as No. 10.	0.38 cc. 17 L	0.38 cc. 16 L++	0.75 cc. 64 D			
12	Same as No. 10.	0.31 cc. 17 L_0	0.38 cc. 16 L++++	0.75 cc. 111 D			
13	Six instillations = 0.15 cc. at intervals of 14 days.	0.38 cc. 16 L_0	0.38 cc. 24 L++	0.38 cc. 75 L_0	1.1 cc. 36 L+++		
14	Same as No. 13.	0.38 cc. 16 L++	0.38 cc. 24 L+++	0.38 cc. 75 L_0	1.13 cc. 101 L		
15	Same as No. 13.	0.38 cc. 16 L_0	0.38 cc. 24 L	0.38 cc. 75 L_0	1.13 cc. 101 L		

dency, elaborated with time, towards a greatly strengthened degree of immunity. Particular attention is called to a comparison of the histories of Guinea Pigs 8 and 9. Both had received essentially the same preparation but Guinea Pig 8 succumbed to the second intravenous injection of 0.38 cc. of serum 14 days after the first. Guinea Pig 9 was kept until 68 days after the first injection and then easily withstood 0.5 cc. of serum. Still more to the point is comparison of the records of Guinea Pigs 13 to 15. Each withstood the third in-

travenous injection of 0.38 cc. of serum without obvious reaction. 36 days later Guinea Pig 13 was given an intravenous injection increased to 1.1 cc. and nearly died. The remaining two animals did not receive their fourth intravenous injection until 101 days after the third; each then withstood with hardly perceptible shock the large amount of 1.13 cc. of serum by the vein. It will be noted that the reactions of Guinea Pigs, 10, 11, and 12 are wholly different from those described above. These animals received a preparatory nasal treatment of twelve instillations repeated at intervals of 24 hours instead of on alternate days. Several diverse experiences have led us to conclude that qualitative differences exist between the immunological response elicited in animals according as they receive successive instillations of serum within one or two or more days.

TABLE III.

The Influence of Time on the Development of Immunity in Guinea Pigs Prepared by Intraperitoneal Injection and Tested by Intravenous Injection of Horse Serum.

No. of guinea pig.	Preparation.	First intravenous injection. 1. Amount of serum. 2. No. of days since last intraperitoneal injection. 3. Result.	1. Amount of serum. 2. No. of days since last intravenous injection. 3. Result.					
			Second intravenous injection.	Third intravenous injection.	Fourth intravenous injection.	Fifth intravenous injection.	Sixth intravenous injection.	Seventh intravenous injection.
1	All animals were given six intraperitoneal injections of serum within a period of 10 days.	0.19 cc. 9 L++	0.2 cc. 22 L++++	0.31 cc. 16 L++	0.31 cc. 19 L+	0.31 cc. 78 L+	0.63 cc. 45 L++	0.63 cc. 116 L+++
2		0.31 cc. 9 L+	0.32 cc. 22 L+	0.38 cc. 16 L	0.38 cc. 19 L+	0.38 cc. 78 L	0.75 cc. 45 L+	0.75 cc. 116 L+++
3		0.2 cc. 32 L++	0.25 cc. 24 L++++	0.25 cc. 12 L	0.75 cc. 94 D			
4		0.2 cc. 32 L+	0.26 cc. 18 L+	0.25 cc. 18 L+	0.38 cc. 94 D			
5		0.23 cc. 32 L++	0.33 cc. 18 L	0.33 cc. 18 L++	0.38 cc. 95 D			

Table III represents the histories of five guinea pigs which received courses of intravenous injections of horse serum after a preparatory period in which six intraperitoneal injections of 0.5 cc. of serum were given within 10 days. Comparing the animals with Nos. 1, 2, and 3 of Table II we see that they were capable of tolerating a much larger initial intravenous injection than the latter, but in Table III the tolerance of the subjects to increasing doses of the antigen is shown to have increased more slowly than in Table II. But the fundamental difference is that the guinea pigs of Table III, after having been made relatively immune to a certain intravenous dosage of serum, when kept for 3 months or more and then reinjected, manifested an increase of susceptibility, whereas in Table II the contrary is the case under similar conditions.

It should be expected that Guinea Pigs 1 and 2 of Table III would have been thoroughly immunized by the long succession of intravenous injections. Nevertheless we find that when a seventh injection was given following a resting period of 116 days after the sixth the animals were much more profoundly shocked by the dose which had previously been fairly well tolerated. They would undoubtedly have succumbed to any such increase of dosage as was employed with impunity on the subjects of Table II.

General Hypersensitiveness Is Not Abolished by Intranasal Treatment.

Since it has been shown that immunity to toxic injections could be established by instillations into the nose of definite, small amounts of horse serum, that is, that intranasal treatment might be used to produce with certainty prophylaxis against anaphylaxis, it was important to discover whether the cure of a condition of serum hypersensitiveness might be effected in a similar manner. Several experiments were undertaken with this end in view in our work already reported. The results were uniformly negative; no animal which had been sensitized by subcutaneous injection of horse serum and was subsequently treated by nasal instillations of serum survived an intravenous injection of 0.25 cc. afterwards. Since learning the fundamental biologic importance of the amount of serum used in the instillations

it was thought well to repeat these experiments. Our results have thus far been uniformly negative, but in view of the practical importance of the subject the investigation is still being pursued.

DISCUSSION.

The results which have been described strengthen the hypothesis on which the work was founded; namely, that the mucous membrane of the nose is an avenue to the mechanism of immunity which offers peculiar advantages over parenteral routes. Nothing less should be expected if, as seems probable, nasal absorption is a normal stage in the development of natural immunity. We have shown that a guinea pig which has been treated by a series of four nasal instillations of 0.04 cc. of horse serum on alternate days may withstand 16 days later the relatively enormous toxic injection of 0.38 cc. with hardly perceptible reaction. It would appear that the instilled serum had not been absorbed by the nose were it not that a second toxic injection given 15 days after the first may likewise be easily tolerated.

Reasons have been given for believing that every application of as much as 0.02 cc. of horse serum to the mucous membrane of the nose of the guinea pig is attended with sufficient absorption to produce systemic effect. The biologic result of this absorption is qualitatively determined by the quantity of serum instilled and by the extent of mucous membrane with which it comes in contact.

The experiments seem to show that a few nasal instillations of serum quantitatively below a fairly definite minimum lead to a general elevation of the threshold of cellular irritability towards the antigen. Instillations of serum quantitatively in excess of a certain minimum induce, on the other hand, the opposite effect and lower the threshold of cellular irritability. In the first case, the advent into the body of an enormous increase in the amount of serum is tolerated with indifference; in the second case, a comparatively small toxic injection sets up vital reactions with a fatal outcome. Furthermore, it has been demonstrated that the direction in which the plane of metabolic irritability is shifted, and according to which the animal's sensitiveness to the antigen is decreased or increased, is determined by the first two to four of a series of separate instilla-

tions. Transferring these conceptions to the field of clinical experience, we find an explanation of many empirically determined truths.

No one will question the necessity of imposing absolute rest upon a member which has suffered an infected wound, an insistence on which has recently been made by Heidenhain.[4] The parting of the way to recovery or death is often marked by the signs of rest or use at the moment of injury. It is obvious that toxic absorption from the site of trauma must be quantitatively in somewhat inverse proportion to the quietude of the infected part.

If we apply to clinical conditions deductions drawn from our experiments with horse serum, the danger to the human organism from local infection lies not in the absorption of a lethal dosage of poison but in the fact that quantities of toxin in the circulation in excess of a certain minimum render the living cells hypersusceptible to the toxin and transform them to a state of disastrous reactivity (allergy). On the other hand, absorption of toxins in amounts below the critical minimum is not biologically indifferent, but progressively strengthens the resistance of the cellular protoplasm against the later onslaught of enormous doses of toxin. In short, we wish to indicate the necessity for rest in therapeusis.

Our experiments indicate that the elevation or depression of the plane of cellular irritability has been determined at the end of a day or two after inoculation with poisonous material; hence the peculiar value of the early application of the rest treatment. Heidenhain and others have pointed out the surpassing importance of rest at the beginning of a course of surgical infection. Abundant as is the clinical endorsement of similar treatment during the prodromal stages of all medical infections, we must express our doubt whether medical practitioners generally apprehend the value of that early quietude on the part of the patient, the therapeutic importance of which we have sought to establish on a rational basis. Medical infections, whether acute or chronic, undoubtedly involve the same principles as those set up by accident or intention. The necessity for rest and exercise as here set forth will find its effective censor in the practical clinician.

[4] Heidenhain, L., *Münch. med. Woch.*, 1915, lxii, 1482.

It may be suggested that in prophylactic vaccination, as against typhoid fever, a new importance is given to the choice of quantity of material employed in the initial dose. Finally, it must be clear that underlying these investigations is the desire to define more accurately the general principles of prophylaxis against infectious disease.

It is not improbable that choice of the nasal route as a channel of protective inoculation would be simply a return to ancient practice; it is said that the Chinese and Hindus long ago vaccinated against smallpox by blowing the powdered virus into the nose.[5] Today literature is beginning to show evidence of desultory use of the nasal mucous membrane as an avenue of inoculation against infection.[6]

We have been strengthened in the notion, suggested in our former paper, that the introduction of an antigen into the organism leads to the development of two antagonistic antibodies, one of which makes for anaphylaxis and the other against it. If this is true, it is evidently the characteristic property of nasal absorption to allow the easy propagation of one antibody in preference to the other. We think we have demonstrated the experimental conditions under which this can be done. It is a familiar fact that normal guinea pigs inoculated with the serum of hypersensitive animals become themselves passively anaphylactic. It may be proper to record here that, in a course of experiments still under way we have found, in cooperation with Mitchell, that the serum of our protected guinea pigs when inoculated into normal animals has, under certain conditions, been able to protect them from the effects of several intravenous injections of the antigen given at 14 day intervals.

SUMMARY.

1. Normal guinea pigs treated by four to six instillations of horse serum into the nose on alternate days become either hypersensitive or refractory to an intravenous injection of 0.38 cc. of serum given 16 days after the last instillation. If the amount of serum in each

[5] Klebs, A. C., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 69.

[6] See Paget, O., *Med. Rec.*, 1915, lxxxviii, 470. Herrman, C., *N. Y. State J. Med.*, 1915, xv, 233.

instillation is as much as 0.2 cc., anaphylactic death is caused by the toxic injection. If the amount of serum in each instillation is reduced to 0.04 cc. the first intravenous injection is without marked effect, and a second injection and subsequent injections of the same amount of antigen are well tolerated in about half the cases.

2. The effect produced by a given dose of serum, whether protective or anaphylactic, depends probably upon the extent of contact with the mucous membrane of the nose.

3. Guinea pigs which, after nasal treatment, have become tolerant to a definite maximum intravenous injection of the antigen appear to increase the degree of their tolerance, at least up to a resting period of more than 4 months. The same does not hold in animals immunized by the peritoneal route.

4. The first two or three instillations of a series probably determine the biologic character, whether of hypersensitiveness or hyposensitiveness, of reaction towards the serum.

5. It is probable that, contrary to the case in parenteral sensitization, hypersensitiveness and protection, respectively, set up by nasal instillations and not followed by parenteral injections, gradually disappear in about 50 to 100 days.

6. We have failed in attempts to eliminate hypersensitiveness, due to subcutaneous injection of serum, by nasal instillations which would protect the normal animal from the development of anaphylaxis.

7. It is suggested that the principles of prophylaxis evolved under these relatively simple conditions should be applied in the study of infectious disease.

CONCLUSION.

We deduce from our observations that the peculiar value of rest in the treatment of infection depends upon the fact that absorption of minimal amounts of toxic matter produces a positive protective reaction in the organism, while the absorption of larger amounts renders the cells hypersensitive. The biologic response to the intoxication is probably chiefly determined within the first 48 hours of absorption, and, therefore, rest at the beginning of an infective process has preponderant prophylactic value.

THE DIFFERENTIATION OF CELLS AS A CRITERION FOR CELL IDENTIFICATION, CONSIDERED IN RELATION TO THE SMALL CORTICAL CELLS OF THE THYMUS.

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PLATES 8 TO 10.

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Means of Cell Identification.

Until recently the morphological structure of a cell has furnished the chief criterion for its identification. Erythrocytes and leukocytes, nerve and muscle cells, and various other specifically differentiated cells exhibit in their structure sufficient characters for their identification.

The identification of groups of different cells connected locally and genetically is more difficult. In the hematopoietic organs of an adult organism, for example, there are located groups of different cells connected by uninterrupted stages of transition. The youngest and the least differentiated cells give rise to more highly differentiated cells, and at the same time increase their number by multiplication. The proliferation of the young stem cells and their simultaneous differentiation in various directions, according to environmental conditions, lead to considerable diversity between cells, coexisting in time and place; and the various stages of different cells may sometimes manifest merely slight and doubtful peculiarities in their structure.

In similar cases histo- and embryogenetic studies may be of assistance in the identification of various cells, and may establish a gradual differentiation of the cells, in accordance with different embryonic stages. They may also connect certain structures with definite external factors, and by this means greatly facilitate the identification of the various cells in the adult organism. There still remain many cases in which the identification of cells is doubtful.

The small round cells of the cortex of the thymus are an example of this.

It has recently been established that the form of the cell is not a characteristic of certain kinds of cells. The method of tissue culture, which gives an opportunity of observing directly the gradual changes, occurring in the cell *in vivo*, shows how unessential the form of the cell is for its inherent potencies, and how completely this form depends upon the physical conditions under which the cell lives.

Harrison[1] has shown that the nerve cells of frogs may be stimulated by solids, that they respond to solids by an orienting movement, that they adapt their form to that of the spider web, and, finally, that they become spherical within a large hanging drop. Uhlenhuth[2] has established the dependence of the form of the epithelial cell upon the density of the medium in which the cell is growing. In a soft medium the epithelial cell, according to Uhlenhuth, assumes a spindle form, which is generally considered a characteristic feature of connective tissue cells. On the other hand, a liquid medium makes the epithelial cell spherical in shape. Burrows[3] connects the shape of the cell with a distribution of metabolic products in the medium. Finally, Rous[4] has shown that the spindle-shaped cells of connective tissue growing *in vitro*, when liberated from the clot by means of trypsin, assume at once the spherical form. Thus the cell form in its latest conception is not a criterion for cell identification.

To what extent, therefore, has our conception regarding specifically differentiated cells to be altered? Is the cell form merely the reflection of the density of the medium or is a certain kind of cell form implied also by the chemical constitution of the cell? There is strong evidence for the assumption that the shape of the cell is the

[1] Harrison, R. G., The Reaction of Embryonic Cells to Solid Structure, *J. Exp. Zool.*, 1914, xvii, 521.

[2] Uhlenhuth, E., Cultivation of the Skin Epithelium of the Adult Frog, *Rana pipiens*, *J. Exp. Med.*, 1914, xx, 614; The Form of the Epithelial Cells in Cultures of Frog Skin, and Its Relation to the Consistency of the Medium, *ibid.*, 1915, xxii, 76.

[3] Burrows, M. T., The Tissue Culture as a Physiological Method, *Tr. Cong. Am. Phys. and Surg.*, 1913, 77.

[4] Rous, P., and Jones, F. S., A Method for Obtaining Suspensions of Living Cells from the Fixed Tissues, and for the Plating Out of Individual Cells, *J. Exp. Med.*, 1916, xxiii, 549.

result of external physical factors. On the other hand, the structural differentiation of the cyto- and karyoplasm seems to depend upon the chemical constitution of the cell and upon the environmental conditions to which the cell is submitted. The specific chemical constitution of the different cell groups is exhibited in their specific morphological structure and is manifested in the resting stage as well as in the mitotic stage. The differences in the constitution of the cells make them non-interchangeable even under equal conditions, at least under those found in the living organism.

The process of cell differentiation under definite conditions is always the same for certain kinds of cells. Therefore the manifestation of further differentiation potentialities by a cell, the nature of which is doubtful, may be used as a criterion for its identification.

The Small Thymus Cells.

The nature of the small thymus cells has long been a subject of controversy. These cells are considered by one group of investigators (Prenant,[5] Bell,[6] Stöhr,[7] and others) as derivatives of the epithelium and capable of again developing into true epithelial cells. In accordance with this conception, the form and the structure characteristic of a definite cell group, by means of which the cell unity of the small lymphocyte was easily identified (in the blood, in the loose connective tissue, in the bone marrow, in the lymph glands, and in the spleen), became an unessential and temporary attribute of cells, in reality entirely unrelated to the small lymphocytes.

Another group of investigators (Maximow,[8] Danchakoff,[9] and

[5] Prenant, A., Recherches sur le développement organique et histologique des dérivés branchiaux, *Compt. rend. Soc. biol.*, 1893, v, 546. Prenant and Saint Remy, Sur l'évolution des formations branchiales chez le lézard et l'orvet, *Compt. rend. Acad.*, 1902, cxxxv, 1.

[6] Bell, E. T., The Development of the Thymus, *Am. J. Anat.*, 1906, v, 29.

[7] Stöhr, P., Ueber die Natur der Thymus-Elemente, *Anat. Hefte*, 1906, xxxi, 407.

[8] Maximow, A., Untersuchungen über Blut und Bindegewebe. V. Über die embryonale Entwicklung der Thymus bie Selachiern, *Arch. mikr. Anat., IIte Abt.*, 1912, lxxx, 39.

[9] Danchakoff, V., Untersuchungen über die Entwicklung von Blut- und Bindegewebe bei Vögeln; das lockere Bindegewebe des Hühnchens im fetalen Leben, *Arch. mikr. Anat.*, 1908–09, lxxiii, 117; Ueber die Entwickelung der embryonalen Blutbildung bei Reptilien, *Verhandl. anat. Ges., Anat. Anz.*, 1910, xxxvii, Suppl., 70.

especially Hammar[10]) established a close connection between the development of the small thymus cells and an infiltration of the thymus by wandering cells, which proliferated and differentiated within the epithelial thymus anlage into small lymphocytes.

Most of the investigators of both groups based their conclusions on the histogenetic principle of the gradual differentiation of one class of cells from the other. Both groups of investigators supported their conclusions by the existence of transition stages between the differentiated small thymus cells and the cells from which they were derived. As a matter of fact, one group referred to transition stages between the small thymus cells and the invading mesenchymal elements, whereas the other mentioned no less definitely the presence of transition stages between the small thymus cells and the cells of the epithelial anlage, overlooking the infiltration of the anlage by wandering cells of mesenchymal origin. The structure of the small thymus cells, identical with that of the small lymphocytes, caused them to appear merely as a disguised form of a true epithelial cell.

The insufficiency of the histogenetic principle in the identification of the small thymus cells was obvious from the fact that in 1908 various workers in the Institute for Histology at Moscow simultaneously expressed opposite views. Gamburtseff[11] considered the small thymus cells to be epithelial elements; Danchakoff[12] established in the thymus of birds a differentiation of the small lymphocytes at the expense of lymphoid hemocytoblasts (large lymphocytes). Maximow's[13] histogenetic studies demonstrated the lymphatic nature of

[10] Hammar, J. A., Der gegenwärtige Stand der Morphologie und Physiologie der Thymusdrüse, *Congrès internat. méd., Budapest, Compt. rend.* Fünfzig Jahre Thymusforschung. Kritische Übersicht der normalen Morphologie, *Ergebn. Anat. u. Entwcklngsges.*, 1909, xix, 1.

[11] Gamburtseff, A., Die Histogenese der Thymus, Dissertation, Moskow, 1908. Cited by Hammar, J. A., *Ergebn. Anat. u. Entwcklngsges.*, 1909, xix, 274.

[12] Danchakoff, V., Untersuchungen über die Entwicklung von Blut- und Bindegewebe des Hühnchens im fetalen Leben, *Arch. mikr. Anat.*, 1908–09, lxxiii, 117.

[13] Maximow, A., Untersuchungen über Blut und Bindegewebe. IV. Über die Histogenese der Thymus bei Amphibien, *Arch. mikr. Anat., 1te Abt.*, 1912, lxxix, 560; V. Über die embryonale Entwicklung der Thymus bei Selachiern, *ibid., 1te Abt.*, 1912, lxxx, 39.

the thymus cells in mammals, amphibians, and selachians; the same was done by Danchakoff[14] in the case of reptiles.

The balance of evidence has of late appeared strongly to support the substitution theory, or the theory of the true lymphatic nature of the small thymus cells. However, this problem has not been conclusively solved.

The recourse to cell differentiation as to a criterion for cell identification is not altogether new. In 1909 Schaffer[15] stated that the transformation of the small thymus cells into plasma cells in the lobules of the thymus during involution of the organ may give proof of their true lymphatic nature.

The fact that the mother cells of the small thymus cells in reptiles also differentiate into numerous granulocytoblasts is accounted for by Danchakoff[16] (1910) by the lymphatic nature both of the small thymus cells and of their mother cells. Maximow also mentions the existence of a slight granulocytoblastic development in the thymus of mammals.

It is known that the small lymphocytes develop in part at the expense of the mesenchymal cells, but they appear chiefly as the result of an intense proliferation of the lymphoid hemocytoblasts. The mesenchymal cells, however, as well as the lymphoid hemocytoblasts, are similarly capable of differentiating under definite conditions into granulocytoblasts and granulocytes (granular leukocytes). This line of differentiation is absent in the epithelial cells.

The mother cells of the small thymus cells manifest distinctly in the normal embryos of reptiles (Danchakoff[16]) their power of differentiating into granulocytoblasts. This line of differentiation is, however, only slightly exhibited in birds. In the connective tissue septa but few granulocytoblasts are seen developing at the expense of lymphoid wandering cells. If by experimental intervention the line of differentiation of the mother cell of the small thymus cells may be inter-

[14] Danchakoff, V., Über die Entwicklung des Blutes in den blutbildenden Organen bei *Tropidonotus natrix*, *Arch. mikr. Anat.*, 1916, lxxxvii, 497.

[15] Schaffer, J., Über Thymus und Plasmazellen, *Zentr. Physiol.*, 1908–09, xxii, 422.

[16] Danchakoff, V., Ueber die Entwickelung der embryonalen Blutbildung bei Reptilien, *Verhandl. anat. Ges.*, *Anat. Anz.*, 1910, xxxvii, Suppl., 70.

changed, if it may be directed, for example, into the granulocytoblastic mode of differentiation (which is characteristic only of true lymphoid elements and never occurs in epithelial cells under conditions existing in the organism), it would follow that the small thymus cells are the offspring of true lymphatic elements and are not derivatives of the epithelial cells.

It is also known that the true small lymphocytes may under certain conditions differentiate into plasma cells, and, in the case of birds, into special wandering cells, characterized by the presence in their cytoplasm of numerous minute acidophylic granules. An intensive differentiation of the cortical cells into plasma cells in the thymus of the hen may be observed after the administration of small doses of x-ray to the animals. This differentiation appears undoubtedly as a proof of the lymphatic nature of the small thymus cells.

Finally, the present study has shown the existence of a physiological differentiation of the small thymus cells into granular lymphocytes, characteristic of birds, which indicates strongly the lymphatic nature of the small thymus cells.

Histogenetically the Small Thymus Cells Are the Offspring of Cells Morphologically Identical with the Lymphoid Hemocytoblasts.

The chief evidence for the lymphoid nature of the small thymus cells was recently shown by an invasion of the epithelial thymus anlage by wandering cells of mesenchymal origin. At the time of the first appearance of the small lymphocytes in the thymus of birds a striking picture of the differentiation processes is seen (Fig. 1). At this time the loose tissue of the thymus medulla merges gradually into the denser cortical layer. The parts of the cortical layer closely adjacent to the medulla appear also less dense than the parts surrounded at the periphery by the loose connective tissue. The cells constituting the cortical layer exhibit a marked difference as regards their structure. In the looser parts adjacent to the medulla numerous large ameboid cells are found, the structure of which is typical for the lymphoid hemocytoblasts (Fig. 1, L. Hbl.). Moreover, many of the cells seem to repeat on a miniature scale the structure mentioned (S. L. Hbl.). The nearer they approach the periphery of the organ,

the denser becomes the tissue and the smaller appear the lymphoid hemocytoblasts. And they tenaciously preserve the characteristic nucleus, which is relatively large, light, and spherical in shape, containing a well defined nucleolus. Though the cytoplasm of these cells diminishes, it still appears intensely basophilic. These cells may be called small lymphoid hemocytoblasts, and multiply profusely. They do not appear specific for the thymus, but arise in the organism where the lymphoid hemocytoblasts proliferate intensely in regions insufficiently supplied with nutritive material. Their appearance in the thymus must therefore be connected with the local agglomeration of the cells in relation to their intensive proliferation.

The new generations of the small lymphoid hemocytoblasts continue to multiply at the expense of the large lymphoid hemocytoblasts as well as by their own mitotic proliferation. The cortical layer of the thymus abounds at this time with mitoses. The decrease in size of the small lymphoid hemocytoblasts does not extend beyond a definite limit. Their further existence in the limited and poorly vascularized space, where the cells are crowded together, must lead to a change in metabolism. This change reveals itself by the differentiation of a new cell form, the small lymphocyte (S. Lmc.). The gradual differentiation of the small lymphocytes at the expense of the lymphoid hemocytoblasts is easily established in the embryonic thymus. A gradual accumulation of differentiated chromatin in the nuclei is discernible in the cells (Fig. 1, x). This accumulation is closely connected with a gradual exhaustion and a final disappearance of the nucleolus. The cells are ameboid and the narrow rim of cytoplasm becomes less basophilic. Fig. 2 shows a variety of transition stages (*a* to *f*) through which a large lymphoid hemocytoblast passes during its transformation into a small lymphocyte.

There is a striking difference in the mitoses (Fig. 3, *a*, *b*, *c*) of the two kinds of cells. It is difficult to establish the number of chromosomes, as their size is very minute. The size and the general aspect of the chromosomes, however, differ widely. The differences between the various kinds of mitosis, specific for certain types of cells, were repeatedly noticed. The peculiarities in the mitosis, appearing in connection with the cell differentiation, must not be overlooked, especially in view of the recent tendency to dissociate entirely the cell differen-

tiation from the factorial background of the cells, which is supposed to be the same in every cell of the body.

In his last book Morgan[17] states: "There is extensive evidence from cytology, experimental embryology, and regeneration, to show that all the different cells of the body receive the same hereditary factors" (which Morgan connects exclusively with the chromosomes). "We must suppose, then, that the Mendelian factors are not sorted out, each to its appropriate cell, so that factors for color go only to pigment cells, factors for wing-shape to cells of the wings, etc., but that differentiation is due to the cumulative effect of regional differences in the egg and embryo, reacting with a complex factorial background that is the same in every cell."

This statement finds support in histogenetic studies and in experimental embryology, but not to the extent outlined above. The hematopoietic studies corroborate the statement quoted above in demonstrating the existence of a lymphoid hemocytoblast, of which further differentiation into various kinds of blood cells is directed by the cumulative effect of regional differences. But there seem to exist in the different cells stages at which the chemical constitution and the metabolism of the cells are changed so essentially that the complex factorial background of the cell ceases to be the same for every cell. From this time on the cells are no longer interchangeable even under identical conditions; as, for example, in the blood stream. Under normal conditions the different kinds of blood cells within the vessels preserve unchanged their structural and functional peculiarities. Most of the cells present in the blood stream have lost their power of multiplication. Yet during leukemias the blood contains numerous less differentiated cells, endowed with mitotic faculty. They nevertheless do not interchange under identical conditions, to which they all are submitted in the blood current. In the hematopoietic organs, before the cells are fully differentiated and have lost their reproductive power, there appear differences in the mitotic process of their multiplication which are specific for certain kinds of cells.

With our limited knowledge and methods we cannot deny the exist-

[17] Morgan, T. H., Sturtevant, A. N., Muller, H. F., and Bridges, C. B., The Mechanism of Mendelian Heredity, New York, 1915, 43.

ence of functional differences between two morphologically identical cells. But from the time that certain groups of cells exhibit, both at the resting stage and during mitosis, well pronounced features characteristic only of these groups, the factorial background of the cell must have changed. If the complex of chromosomes, with which the Mendelian School exclusively associates the different factors, becomes visibly changed, a change in the factors of differentiation may be assumed. In view of a simultaneous restriction of differentiation potentialities in such cells a segregation of the differentiation factors may be assumed, if these factors are really as closely connected with the chromosomes as the Mendelian School supposes.

The establishment of a definite morphological structure for the small lymphocytes at rest as well as during mitosis seems to be an argument for an admission of a special differentiation of this cell unity. The small lymphocytes as such do not present the last stage in their evolution, developing under certain conditions into mast cells, into plasma cells, and into granular cells specific for birds. The analysis of the origin of the small cortical cells in the embryonic thymus shows that these cells must be regarded as derived from stem cells the structure of which is similar to that of the lymphoid hemocytoblasts.

The Mother Cell of the Small Thymus Cell Is a True Lymphoid Hemocytoblast. It Is Polyvalent and May Be Directed into the Line of Granulopoiesis.

The small thymus cell is looked upon by many histologists as a product of differentiation of the epithelial cells. The morphological structure of the small thymus cells, identical with that of the small lymphocytes of the blood stream and lymphatic organs, does not seem to be a sufficient argument to establish the true lymphatic nature of the thymus cells. Therefore it would be vain to suppose that the morphological structure of their mother cells, identical with that of the lymphatic hemocytoblasts, would be taken into account for the solution of the problem. The small thymus cells are considered by many investigators as being merely a disguised form of epithelial cell. The same assumption may be applied to their mother cells.

Stöhr[18] and Marcus[19] believe the small thymus cells to be capable of differentiating into true epithelial cells. Therefore the solution of the problem would depend upon the possibility of adding to the existing criteria new ones that are qualitatively different.

The study of hematopoiesis in reptiles has already given data relative to the potentiality of double differentiation which exists in the mother cells of the small thymus cells. These stem cells develop on the one hand into the small lymphocytes, and on the other hand into granulocytoblasts, and further into granulocytes (granular leukocytes). In birds and mammals this process does not seem to be of any importance. The polyvalence of the stem cells, which, in the thymus of reptiles, differentiate into cortical cells and granular leukocytes, is a strong argument for their true lymphatic nature and consequently for the lymphatic nature of both kinds of their daughter cells. If there is doubt of the identification of certain kinds of cells and of their mother cells, but if a possibility exists of tracing a characteristic line of differentiation of the polyvalent mother cells, the nature of these common mother cells is thereby solved and the identification of the other products of differentiation greatly facilitated.

On a recent occasion I had the opportunity[20] of demonstrating the polyvalence of the mesenchymal cells in embryos of 7 to 10 days, which revealed itself in a striking manner through the whole loose mesenchyme after spleen grafts on the allantois.[21] The specific line of granulocytoblastic differentiation, exhibited by the mesenchymal cells in different regions of the body, is also shown by the lymphoid hemocytoblasts of the thymus.

The anlage of the thymus appears in the form of separate foci of epithelial tissue, which at the beginning are independent. These agglomerations of epithelial cells develop in a full grown bird into large flat glands (0.5–1.5 cm. in length) which often join. They are located in the embryo, as well as in the adult animal, a little be-

[18] Stöhr, P., Ueber die Natur des Thymuselemente, *Anat. Hefte*, 1906, xxxi, 407.

[19] Marcus, H., Ueber die Thymus. Lebenslauf einer Thymuszelle, *Verhandl. anat. Ges., Anat. Anz.*, 1907, xxx, Suppl., 237.

[20] Meeting of the Association of American Anatomists, New Haven, Conn., Dec. 28–30, 1915.

[21] Murphy, Jas. B., *J. Exp. Med.*, 1916, xxiv, 1.

hind the neurovascular bundle of the neck, and extend a little lower than the clavicle.

Macroscopically the grafting is followed by a visible enlargement of the thymus glands. This enlargement may be noticed as early as 2 days after grafting. On microscopic examination the thymus appears to be widely infiltrated by the large lymphoid hemocytoblasts, which proliferate intensely. The tissue of the thymus assumes a looser appearance, the limits between the cortex and the medulla disappearing almost completely. The infiltration by hemocytoblasts is equally strong in the cortex and medulla. Even at this early stage, after grafting, the lymphoid hemocytoblasts show conspicuously a tendency to differentiate in a granulocytoblastic direction and many of them develop in their cytoplasm numerous acidophylic granules. This line of differentiation of the hemocytoblasts is intensely developed in the thymus tissue 3 to 4 and 5 days after grafting (Fig. 4). One of the numerous protuberances covering the surface of the thymus is shown in the drawing. On the right of the figure is seen a part adjacent to the medulla. On the left are shown the peripheral layers of the thymus.

The cells forming the tissue of the thymus are different in structure. Besides a few reticulum cells, the cortical layer of the thymus contains large lymphoid hemocytoblasts (L. Hbl.), characteristic of this stage, and also small lymphocytes (S. Lmc.), which develop at the expense of the first mentioned cells. The differentiation of the small lymphocytes is, however, not intense, as in the normal thymus of the same stage (Fig. 1), and seems to be partly substituted by an intense development of granulocytoblasts and granulocytes (Fig. 4, Grbl., Grc.). This process of differentiation of the large hemocytoblasts undoubtedly corresponds to what is seen normally in the specific granulocytoblastic organs, chiefly in the annexes of the yolk sac (Danchakoff[22]), and in the bone marrow, and partly in early stages in the mesenchyme.

[22] Danchakoff, V., Untersuchungen über die Entwickelung des Blutes und Bindegewebes bei den Vögeln. I. Die erste Entstehung der Blutzellen beim Hühnerembryo und der Dottersack als blutbildendes Organ, *Anat. Hefte*, 1908, xxxvii, 471; Ueber die Blutbildung im Dottersack des Hühnchens, *Verhandl. anat. Ges., Anat. Anz.*, 1908, xxxii, Suppl., 72.

The development of the granulocytoblasts and their further differentiation into granular leukocytes is principally localized in the parts of the thymus where the tissue is looser. The tissue being less dense in the medulla and in the adjacent parts of the cortical layer, more numerous granulocytoblasts develop. The hemocytoblast here seems to find suitable conditions for growing into a large cell and for developing in its cytoplasm numerous acidophylic granules. A peculiar feature in the differentiation of granulocytoblasts after grafting is exhibited by pronounced differences in the size of the cells. It is possible that this is connected with the intensity of the process, spreading out over many cell generations at the same time. Most of the granulocytoblasts here are also large cells. The cells in Fig. 5, *a* to *f*, show the gradual transformation of a lymphoid hemocytoblast into a granulocytoblast and further into a granulocyte. The proliferation of the cells in the peripheral layers of the thymus is very intense, as is normally the case, and the hemocytoblasts gradually become smaller. The areas of rapid cell proliferation, accompanied by a diminution in cell size, are here, as normally, the first centers of differentiation of the small lymphocytes.

The transformation of the stem cells in the thymus into granulocytoblasts is an important argument for their true lymphatic nature and also for the lymphatic nature of the other products of differentiation of these stem cells; namely, of the small thymus cells. The same stem cells which under conditions of normal development produce a generation of small thymus cells now become the source of a most intense granulopoiesis. It is known that in normal development granulocytes and granulocytoblasts differentiate at the expense of a polyvalent lymphoid hemocytoblast, which under definite conditions becomes also the source of development of small lymphocytes.

The possibility of experimental deviation of the normal development of the mother cells in the thymus proves: (*a*) that the mother cells of the small thymus cells, being capable of differentiation also into granulocytoblasts, are true lymphoid hemocytoblasts; and (*b*) that the small thymus cells, being the offspring of true lymphoid hemocytoblasts, and exhibiting the structure of small lymphocytes, are true small lymphocytes.

That the mesenchymal cells and the lymphoid hemocytoblasts

developing from them are known to be polyvalent, is chiefly the result of the studies of Bryce,[23] Danchakoff, and Maximow. The study of hematopoiesis in birds and reptiles has shown that the granulo- and erythropoiesis, both developing from a common source, proceed independently under different environmental conditions (Danchakoff[24]). By this means the form and the finer structure of cells, derived from the polyvalent mesenchymal cells were thought to be the result of definite chemicophysical influences. The mesenchymal cells and the hemocytoblasts do not escape, under the conditions of the present experiments, the influence of the chemicophysical agents. The granulocytoblasts as usual develop from hemocytoblasts outside the vessels, and the hemocytoblasts derived from the mesenchymal cells partly differentiate into small lymphocytes, as is normally the case. The experiments confirm the statement that the differentiation is dependent upon the environmental conditions. The proliferation, however, seems to be influenced by specific stimuli which also enable a partial deviation of the normal differentiation.

The stimuli for the proliferation of the cells in the loose mesenchyme, which are strong in the early periods of embryonic development, decline in later stages, and the proliferation of these cells stops in certain parts of the organism and proceeds very slowly in others. These cells may be regarded as being "in a period of rest from which they may be aroused by special substances" (Loeb[25]). After grafting, the presence in the circulation of specific products of metabolism from the grafted spleen cells produces a strong irritation in the mesenchymal cells. The mesenchymal cells respond to this irritation by growth,

[23] Bryce, T. H., The Histology of the Blood of the Larva of *Lepidosiren paradoxa*. Part I. Structure of the Resting and Dividing Corpuscles, *Tr. Roy. Soc. Edinburgh*, 1904, xli, 291; Part II. Hæmatogenesis, *ibid.*, 1905, xli, 435.

[24] Danchakoff, V., Untersuchungen über die Entwickelung des Blutes und Bindegewebes bei den Vögeln. I. Die erste Entstehung der Blutzellen beim Hühnerembryo und der Dottersack als blutbildendes Organ, *Anat. Hefte*, 1908, xxxvii, 471; Ueber die Blutbildung im Dottersack des Hühnchens, *Verhandl. anat. Ges., Anat. Anz.*, 1908, xxxii, Suppl., 72; Ueber die Entwicklung des Knochenmarks bei den Vögeln und über dessen Veranderungen bei Blutentziehungen und Ernährungsstörungen, *Arch. mikr. Anat.*, 1909, lxxiv, 855.

[25] Loeb, J., The Stimulation of Growth, *Science*, 1915, xli, 704.

multiplication, and differentiation, which in this case is into granulocytoblasts and granulocytes.

Loeb[26] attributes the stimulation of growth to the influence of certain substances. Gudernatsch[27] has shown that the feeding of thyroid substance has a stimulating effect upon the growth of the limbs of the tadpole. In the latter case the thyroid substance merely accelerates the normal rate of proliferation of embryonic cells.

The sudden and general proliferation of the mesenchymal cells and their further differentiation in the embryo body after grafts on the allantois includes also cell groups which would normally remain indefinitely in a period of rest. This sudden proliferation is accompanied by a differentiation, which would also not have taken place here. In this respect the results of my experiments correspond closely to those of Loeb in connection with the regeneration in *Bryophyllum calycinum*. Only the wound stimulus is absent in the experiments with grafting, and the proliferation and differentiation of the mesenchymal cells in the embryo body are probably stimulated by the appearance in the embryonic tissues of specific substances, involving the growth of certain kinds of cells. This power of stimulating the growth at will and of directing the differentiation of the mesenchymal cells may offer further suggestions in the study of hematopoiesis.

For our partial problem the deviation of the normal differentiation of the polyvalent stem cells in the thymus into another line seems to me to be definite proof of the true lymphatic nature both of the stem cells and of the small cortical cells.

The Small Thymus Cell Is a True Small Lymphocyte. It May Differentiate into a Granular Lymphatic Cell and into a Plasma Cell.

The various potentialities of differentiation in a polyvalent mother cell may under definite conditions be used for the identification of the daughter cells. The further differentiation of the daughter cells may also serve as criteria for cell identification.

[26] Loeb, J., Rules and Mechanism of Inhibition and Correlation in the Regeneration of *Bryophyllum calycinum*, *Bot. Gaz.*, 1915, lx, 249.

[27] Gudernatsch, J. F., Feeding Experiments on Tadpoles. II. A Further Contribution to the Knowledge of Organs with Internal Secretion, *Am. J. Anat.*, 1914. xv, 431.

The tissue of birds, particularly the loose connective tissue, contains characteristic wandering cells, which show in their cytoplasm numerous small spherical eosinophilic granules. These cells develop at the expense of the small lymphocytes in later stages of embryonic life (Danchakoff), and their presence in the connective tissue of the adult animal has been described by Solucha.[28] The present study has shown that this process of differentiation of specific granular lymphocytes also takes place physiologically in the normal thymus glands. Fig. 6 represents a small group of such cells in the loose connective tissue and Fig. 7 a group of similar cells in the thymus. The cell marked x shows the gradual accumulation of small eosinophilic granules.

A development of the small thymus cells into another line of differentiation, namely, into plasma cells, may be induced by administration to the animals of small doses of x-ray. Schaffer described analogous changes of small lymphocytes into plasma cells as occurring during the physiological involution of the thymus. Numerous small thymus cells differentiate under these conditions into plasma cells. Fig. 8 shows a group of developing plasma cells situated in the reticulum. It is easy to follow the gradual development of a plasma cell from a small thymus cell (S. Lmc.). The cytoplasm becomes more abundant and basophilic and the nucleus undergoes characteristic changes (x). The chromatin condenses into larger blocks which usually adhere to the membrane. This change leads finally to the formation of a typical *Radkern*.

Normal thymus glands, excised before the treatment of the animals, show that the occasional differentiation of the small thymus cells into plasma cells is due to the specific influence of small doses of x-rays.

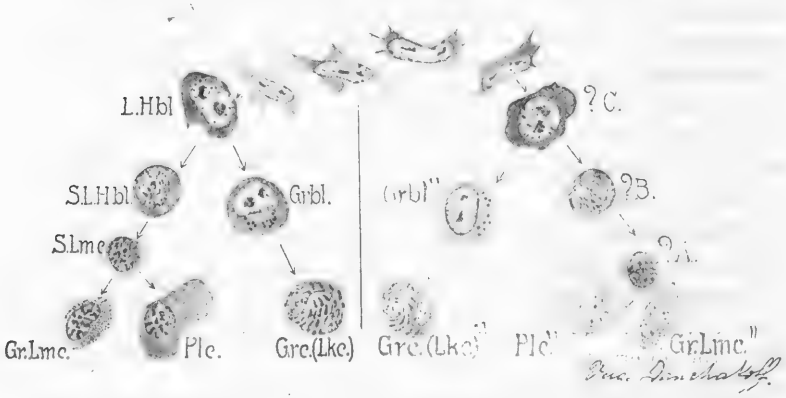
The experimental and physiological transformation of the small thymus cells and their mother cells into products peculiar only to the group of lymphoid elements thus causes the small thymus cells and their mother cells to be included in this group.

It is important in this connection, that the various differentiations described above are confined under the conditions cited, both to the thymus cells and their stem cells, as well as to the true small lymphocytes and the lymphoid hemocytoblasts found in other parts of the organism. The differentiation of the hemocytoblasts into granulo-

[28] Solucha, N. P. The Different Kinds of Cells in the Connective Tissue, Dissertation, St. Petersburg, 1908.

cytoblasts and granulocytes has already been mentioned. The physiological differentiation of the small lymphocytes into granular lymphocytes, and the experimental differentiation of the small lymphocytes into plasma cells is equally general.

The principle of differentiation of cells as a criterion for cell identification was considered in the present paper in relation to the small cortical cells of the thymus. The same principle may serve as a criterion for the identification of other cells, the morphological structure of which does not offer sufficiently definite data for their identification.



TEXT-FIG. 1. Scheme for some of the differentiation products of the loose mesenchyme.

SUMMARY.

The main problem which has been considered in this paper is the identification of the small thymus cells. Cells resembling ?A infiltrate the cortical layer of the thymus. Whether these cells are true lymph cells, or whether they merely represent a disguised epithelial cell, could not be solved either by purely histological or by histo- and embryogenetic studies.

Text-fig. 1 gives a scheme for the differentiation of the small thymus cells and the small lymphocytes, which led to a conception of the lymphoid nature of the small thymus cells. On the left of the figure is shown the origin and the normal differentiation of the small

lymphocytes and their mother cells in birds. The source of the lymphoid cells is the loose mesenchyme. The small lymphocytes (S. Lmc.), normally situated in the spleen, in the connective tissue, and in the bone marrow, appear as differentiation products of the lymphoid hemocytoblasts (L. Hbl.), especially when the latter multiply intensely in a limited space, become smaller (S. L. Hbl.), and finally change their morphological structure. The small lymphocytes may themselves under definite conditions undergo further differentiation and develop into plasma cells (Plc.) and into granular lymphocytes (Gr. Lmc.), specific for birds. The mother cells of the small lymphocytes, the lymphoid hemocytoblasts, on the other hand, differentiate in granulopoietic organs (spleen, bone marrow) into granulocytoblasts (Grbl.) and granulocytes (Grc. (Lkc.)).

The histogenetic study of cell ?A shows that its nearest stem cells are represented by the cells ?B and ?C (page 93). The striking similarity in the structure of cell ?A with that of the small lymphocyte (on the left of the text-figure), as well as the analogy of the process of its differentiation, at the expense of cells ?B and ?C, with the normal origin of the small lymphocytes, has led many investigators to accept the mesenchymal origin of the small thymus cells. According to this view, cell ?A becomes a true small lymphocyte. In addition to this is the observation of an invasion of the epithelial thymus anlage by mesenchymal elements.

According to the transformation theory cell ?A has become a disguised epithelial cell. The same was admitted for their stem cells ?B and ?C. As mentioned above, many characteristic morphological features of cell structure were shown to be not as essential as previously admitted. Nerve cells and epithelial and connective tissue cells may all assume a spherical shape in a liquid medium. How then are we to regard the small thymus cell ?A ?

If the mother cell of the thymus cell ?A is really a lymphoid hemocytoblast, it must possess the differentiation potentialities of a true lymphoid hemocytoblast; it must have the faculty of differentiating not only into small lymphocytes but also into granulocytoblasts (Grbl.″) and further into granulocytes (Grc. (Lkc.)″). If the small thymus cell ?A is a small lymphocyte it must under definite conditions be able to differentiate in the directions characteristic of true small

lymphocytes; namely, into plasma cells (Plc.″) and into granular lymphocytes (Gr. Lmc.″). The existence of these possibilities for differentiation of the small thymus cells ?A and their stem cells ?C have been experimentally proved by this study.

It has been shown that the mother cell ?C may under certain conditions proliferate intensely and differentiate into granulocytoblasts and granular leukocytes. This is probably due to the stimulating action of certain metabolic products of the grafted spleen cells.

It has been also shown that the small thymus cell ?A may differentiate physiologically into a granular lymphocyte, and under administration of x-ray into a plasma cell.

All the changes observed on the small thymus cells and their mother cells apply under equal conditions to the lymphoid hemocytoblasts and to the true small lymphocytes in the regions of the organism where these cells are present.

EXPLANATION OF PLATES.

All the figures were drawn with the camera at stage level, with Zeiss Apochromat 2 mm. oil immersion objective, and No. 12 compensating ocular.

The abbreviations are the same for all the figures. Fb., fibroblast; Grbl., granulocytoblast (myelocyte); Grc., granulocyte (leukocyte); Gr. Lmc., granular lymphocyte; L. Hbl.; lymphoid hemocytoblast (large lymphocyte); Msc., mesenchyme cell; Rtcc., reticulum cells in the thymus; S. L. Hbl., small lymphoid hemocytoblast; S. Lmc., small lymphocyte; S. Lmc.′, mitosis of small lymphocyte; x, a transition cell between two stages of differentiation; Plc., plasma cell.

PLATE 8.

FIG. 1. Cortical layer of a normal thymus from a 12 day embryo; intensive development of small lymphocytes at the expense of the lymphoid hemocytoblasts, L. Hbl., with intermediate stages of small lymphoid hemocytoblasts, S. L. Hbl., and transition stages, x.

FIG. 2. Cells showing the gradual differentiation of a small lymphocyte, *f*, at the expense of a large lymphoid hemocytoblast, *a*, with intermediate stages, *b*, *c*, *d*, *e*.

FIG. 3. Mitotic figures; *a* and *b* of a lymphoid hemocytoblast, *c* of a small lymphocyte.

PLATE 9.

FIG. 4. Cortical layer of a thymus of a 12 day embryo after grafting. Differentiation of the lymphoid hemocytoblast into two various directions; into

small lymphocytes (S. Lmc.), into granulocytoblasts (Grbl.), and into granulocytes (Grc.).

FIG. 5. Cells showing the gradual differentiation of a granular leukocyte, *f*, at the expense of a lymphoid hemocytoblast, *a*, with intermediate stages *b*, *c*, *d*, *e*.

PLATE 10.

FIG. 6. A group of granular lymph cells in the loose connective tissue.

FIG. 7. A group of granular lymph cells in a normal thymus.

FIG. 8. A group of developing plasma cells in a thymus after administration of x-ray.

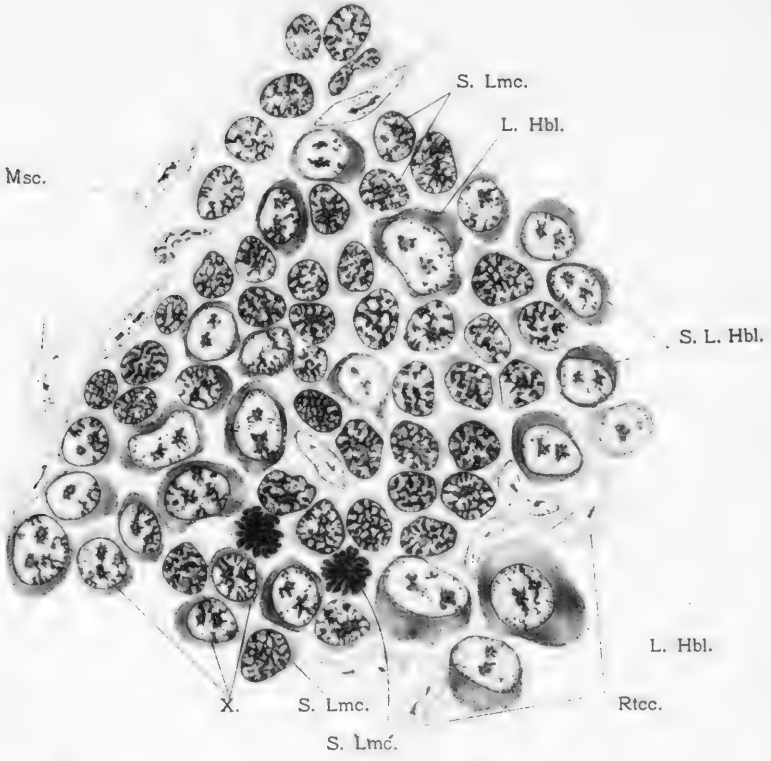


FIG. 1.

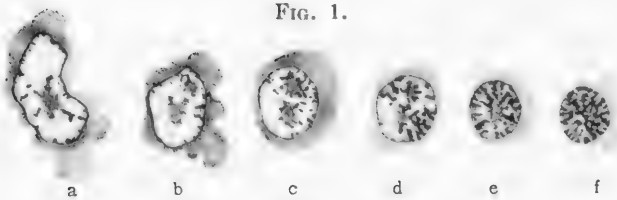


FIG. 2.

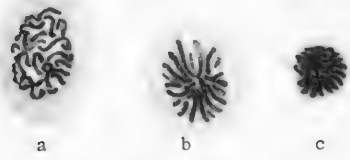


FIG. 3.

Vera Danchakoff fec. (Danchakoff: Differentiation of Cells.)

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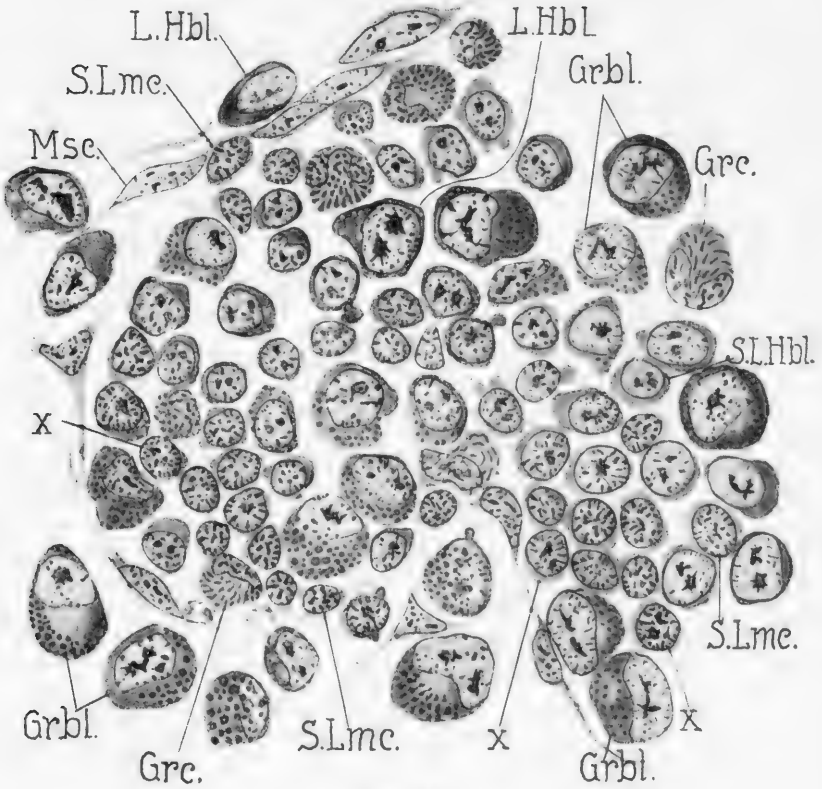

FIG. 4.

FIG. 5.

(Danchakoff: Differentiation of Cells.)

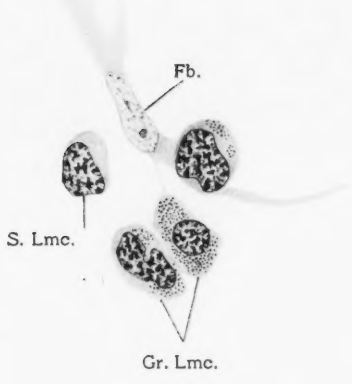

FIG. 6.

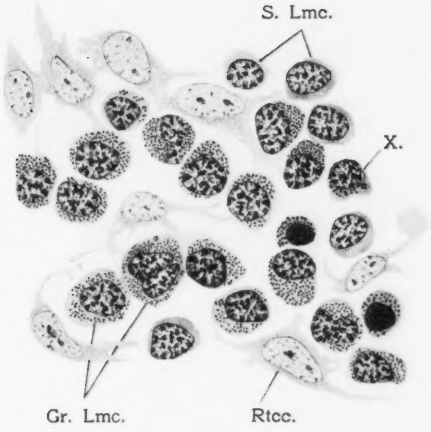

FIG. 7.

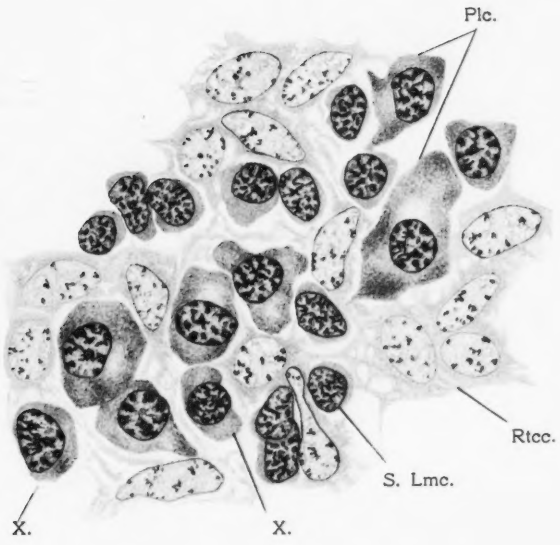

FIG. 8.

Vera Danchakoff fec. (Danchakoff: Differentiation of Cells.)

HELIOTYPE CO., BOSTON